

**A study of the activity and characteristics of
superoxide dismutase
in the male reproductive parts of petunia**

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ABBREVIATIONS

ABA	abscisic acid
ACC	1-amino cyclopropane-1-carboxylic acid
ANOVA	analysis of variance
AOS	active oxygen species
APX	ascorbate peroxidase
Bis	N-N'-methylene-bis-acrylamide
BSA	bovine serum albumin
CAT	catalase
cDNA	complementary deoxyribose nucleic acid
Ca ²⁺	calcium ions
CO ₂	carbon dioxide
Cu ²⁺	cupric ions
Cu/Zn SOD	cuprozinc superoxide dismutase
Cyt	cytochrome
Da	daltons
DEAE	diethylaminoethyl
DH ₂ O	distilled water
EDTA	ethylenediaminetetraacetic acid
Fe ²⁺	ferrous ions
Fe ³⁺	ferric ions
Fe ⁴⁺	ferryl ions
Fe SOD	iron superoxide dismutase
GAPs	guanosine triphosphatase activating proteins
GR	glutathione reductase
GSH	glutamyl cysteinyl glycine
GTPase	guanosine triphosphatase
GUS	β-glucuronidase
HCl	hydrochloric acid
H ₂ O ₂	hydrogen peroxide
HO ₂ [•]	perhydroxyl radical
IMAC	immobilized metal affinity chromatography
KCN	potassium cyanide
kDa	kilodaltons
KOH	potassium hydroxide
L-DOPA	levo-dihydroxy phenylalanine
LM	light microscope
MES	(N-mopholino) ethane sulphonic acid
Mn SOD	manganese superoxide dismutase
MnCl ₂	manganese chloride
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	nicotinamine adenine dinucleotide phoshate

NBT	nitro blue tetrazolium
NO	nitric oxide
NOX	nicotinamine adenine dinucleotide phosphatase oxidase
OH [•]	hydroxyl radical
O ₂ ^{•-}	superoxide anion
¹ O ₂	singlet oxygen
PA	phosphatidic acid
PAGE	polyacrylamide gel electrophoresis
PCD	programmed cell death
PEG	polyethyleneglycol
pI	iso-electric point
PI3K	phosphatidylinositol 3-kinase
PI3P	phosphatidylinositol 3-phosphate
PM	plasma membrane
ROP	a plant specific class of small guanosine triphosphatases
ROS	reactive oxygen species
RNA	ribonucleic acid
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	standard error
SNAP	s-nitroso-acetylpenicillamine
SOD	superoxide dismutase
<i>Sod</i>	DNA or RNA encoding superoxide dismutase
SO ₂	sulfur dioxide
TEMED	N, N, N, N-tetramethylethylenediamine
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
T-test	Tukey HSD All-Pairwise Comparisons Test
UV	ultraviolet light
v/v	volume per volume
w/v	weight per volume

ABSTRACT

In the stamen (male reproductive tissue) of petunia 'Hurrah' flowers, the occurrence of SOD (superoxide dismutase) provided an effective anti-oxidative mechanism against superoxide production. Superoxide production and SOD activities at five developmental stages showed a positive correlation. The highest superoxide production and SOD activity in different parts of the stamen (anther, filament and pollen) were at stages with high metabolic activity: (i) during growing buds (in anthers and filaments) (ii) when flowers with predehiscent anthers were fully open (in pollen). In all parts of the stamen, SOD activity was the lowest at stage five (fully open flowers with dehiscent anthers), superoxide production was also lower at this stage with the exception of the pollen. The highest SOD activity was localized in anthers with the pollen, suggesting that the filaments only have a structural support function.

SOD was examined on a native PAGE with regard to the isozymes present within the stamen of five developmental stages. Three isozymes, which were identified as Mn SOD, Fe SOD and Cu/Zn SOD by reactions with inhibitors, were commonly found at five developmental stages in crude extracts of anthers, filaments and pollen. The developmental stages with stronger isozyme bands on the native PAGE were consistent with the stages with higher SOD activities, and the Mn SOD and Fe SOD isozyme bands were more intense than Cu/Zn SOD bands, suggesting the activities of Mn SOD and Fe SOD in the crude extracts were much higher than Cu/Zn SOD.

SOD from 1,000 stamens of dehiscent mature flowers was partially purified using ammonium sulphate fractionation and DEAE cellulose column chromatography. The purified bound fraction contained only one SOD isozyme on a native PAGE, which was shown to be a Mn SOD, as it is sensitive to neither hydrogen peroxide nor cyanide. The specific activity of the purified SOD was 66.5 U/mg and the yield of total activity was 3.0%. The progress of enzyme purification was monitored using SDS-PAGE and the bound fraction contained two major polypeptide bands. The purified enzyme activity was optimal in the range of neutral pH, but it was the highest at pH 7.8. Through incubation at various pH levels for 24 hours, favourable stability of the purified fraction was confirmed around a pH range of 7 to 8.5. The purified enzyme retained 87% of its initial activity at -20°C after one month of storage, but at 4°C only 38% of the initial activity remained after the same period of storage.

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CHAPTER 1

INTRODUCTION

1.1 General Introduction

Plants in their natural environment are exposed to a variety of stresses as a result of pollutants, changes in light intensities, herbicides, drought, temperature variations, nutritional restrictions and metal accumulation from industrial and agricultural practices. To minimize the effect of these stresses, plants have developed effective mechanisms for adapting to alterations in their environment. In many plant species, these adaptive mechanisms take the form of an ability to combat the presence of reactive oxygen species (ROS), which are produced in a number of different ways during natural metabolic processes in their tissues (Fridovich 1995).

An enzyme called superoxide dismutase (SOD) plays a particularly key role in cellular defences of plants against reactive oxygen species. As plants cannot move around to escape or diminish environmental changes, many have superoxide dismutase as a part of their dedicated defence mechanisms against these changes. This is because superoxide dismutases are metalloproteins catalyzing the dismutation of the superoxide free radical to molecular oxygen and H_2O_2 .

As a result of their relevance to the way human bodies respond to environmental stress, the activities and changes of SOD in response to environmental stresses have been extensively studied in animals, plants and microbes. There has not been, however, as much specific work on SOD activity in the reproductive structures of plants. This is partly because it has been hypothesized that these reproductive organs have developed physical structures that offer good protection against the noxious effects of free radicals and, thus, through this mechanism, avert harm to genetic information (Oden *et al.* 1992). It is therefore relevant to examine this part of the flower to see if superoxide dismutase also plays the same role in

combating reactive oxygen species that it does in other parts of these organisms. Furthermore, this research may also have some relevance to the health of humans, as pollen and pollen extracts have long been used by humans for their anti-inflammatory effect. To this end, McCord (1974) found that exogenous superoxide dismutase protects hyaluronate against depolymerization by free radicals and also indicated that superoxide dismutase might have an anti-inflammatory effect. Unfortunately, there have been very few other papers published on the presence and effect of SOD in pollen. There has, however, been a study that identified two Mn SODs and three Cu/Zn SODs in the pollen grain of maize (Acevedo and Scandalios 1990). SOD activity has also been detected in camelia (Xiao-hong *et al.* 2005) and olive pollen (Alche *et al.* 1998).

In this study, however, the activity and characteristics of SOD in the male reproductive parts of petunia, and purification of this particular SOD, were investigated. This is partly because petunias are popular summer flowering annuals for display purposes throughout the world, but mainly because they are good experimental systems for flower biology investigations.

1.2 Oxidative stress and reactive oxygen species

1.2.1 Reactive oxygen species (ROS) formation and activity

Reactive oxygen species (ROS) contain two free radical species - the superoxide anion ($O_2^{\cdot-}$) and the perhydroxyl radical (HO_2^{\cdot}) - the uncharged, non-radical species hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical (OH^{\cdot}). ROS also include singlet oxygen (1O_2), and the primary source of this is the chlorophyll pigments relevant to the electron transport system. Reactive oxygen species are produced by several diverse mechanisms: through the interaction of ionizing radiation with biological molecules, as an inevitable by-product of cellular respiration, and through being synthesized by dedicated enzymes like NADPH (nicotinamide adenine dinucleotide phosphate) oxidase (NOX) and cell wall peroxidases (Bolwell *et al.* 2002). Intense oxidants, like the diverse ROS, can damage other molecules and the cell structures, and this may result in metabolic

destruction and disrupt cellular structures. It is important to note, however, that attempts to limit the production of ROS would not be desirable, because ROS have some positive functions in plant physiology beyond their role as agents of cellular damage. ROS, at low concentrations, have the function of centrally co-ordinating cell biology and the responses of the latter to a number of environmental stimuli (Desikan *et al.* 2005). For example, macrophages and neutrophils must generate ROS in order to kill some types of bacteria as part of a process of engulfing them by phagocytosis (Halliwell and Gutteridge 1985). Furthermore, abiotic stresses such as photosensitizing toxins, excess irradiation, drought and inappropriate temperatures can destroy cellular homeostasis and increase ROS generation in cells (Alscher *et al.* 2002). It is because of these circumstances that necessitate ROS production that plants have developed a wide range of antioxidant mechanisms to prevent ROS from reaching fatal levels and thus maintain their cellular redox balance and normal metabolism.

1.2.2 Superoxide production

Superoxide is produced in plants in diverse ways, and in several cellular compartments. These ways include non-enzymatic mechanisms, such as electron transfer to molecular oxygen during photosynthesis and respiration in chloroplasts and mitochondria (Mittler 2002). Bowler *et al.* (1994) have identified that the locations of SODs in cells are the same as the three major sites of superoxide production: mitochondria, chloroplasts and cytosol. Plant cell can also increase their generation of superoxide through specific enzymes, such as NADPH oxidases and cell wall peroxidases (Bowell *et al.* 2002).

1.2.2.1 Superoxide production in plasma membranes

Plants, like animals, generate ROS when they are attacked by pathogens (the oxidative burst), as this can provide a direct defence by damaging the pathogen. ROS have also been proposed as having a role in programmed cell death (PCD) and in senescence. Moreover, ROS are thought to be involved in PCD that occurs as part of natural development. Plasma membrane localized NADPH oxidase (NOX) enzymes, producing extracellular superoxide, have been shown to be related to these processes of plant development, pathogen defence, PCD and senescence (Jones and Smirnoff 2005).

It can be seen, therefore, that ROS have a crucial function as signalling molecules in cell growth, development and PCD (Sauer *et al.* 2001), and an important source of these signals is the superoxide ($O_2^{\cdot-}$) generating plasma membrane (PM) NOX complex (Babior 1999). This is because $O_2^{\cdot-}$ can easily generate other ROS, including H_2O_2 and OH^{\cdot} , and because H_2O_2 generation is catalysed by SOD (Halliwell and Gutteridge 1999). Plant NOXs are inherent proteins in the plasma membrane (Keller *et al.* 1998) that catalyse the production of $O_2^{\cdot-}$ from molecular oxygen using reduced NADPH as an electron donor (Sagi and Fluhr 2001).

Regular NOX-mediated $O_2^{\cdot-}$ production in the PM of plants is regulated by Ras small GTPase (ROP GTPase), Ca^{2+} , phosphatidic acid and PI3P (phosphatidylinositol 3-phosphate). NADPH oxidase, containing six transmembrane domains, predicts N- and C-terminal cytoplasmic domains, which are activated by Ca^{2+} *in vitro* because the N-terminus has one or two Ca^{2+} -binding motifs. Therefore, Ca^{2+} may be a major factor in regulating NOX-mediated $O_2^{\cdot-}$ production in plants. This oxygen reduction occurs on the apoplasmic side. H_2O_2 can activate (during PCD) or inactivate (during hypoxic response) NOX in a feedback mechanism. ROP GTPases, in the active GTP-bound form, are also expected to activate NOX, and this is reversed by ROP GTPase activating proteins (ROP-GAPs). In addition, ABA-induced ROS formation is diminished by phosphatidylinositol 3-kinase (PI3K) inhibitors, which prevent synthesis of PI3P, and by PI3P binding proteins. Finally, ROS formation and PCD are also stimulated by phosphatidic acid (PA) in a ROP-dependent manner (Jones and Smirnov 2005).

1.2.2.2 Formation of superoxide in plant cell walls

The production of $O_2^{\cdot-}$ is regulated by cell wall-bound peroxidases and cell membrane-bound NADPH oxidases. When $O_2^{\cdot-}$ is formed in plant cell walls, wall-bound peroxidase first has to be activated by H_2O_2 so as to transform the peroxide (with Fe^{3+}) into what is known as compound I (where the iron is oxidized to Fe^{4+} and then the enzyme bound to an oxygen atom). Once this occurs, compound I can oxidize NADH to an NAD^{\cdot} radical, simultaneously changing itself into what is called compound II (with the iron still in the form of Fe^{4+}). Next, compound II oxidizes another NADH to NAD^{\cdot} , and this reaction converts the peroxidase back into its Fe^{3+} ground state. The NAD^{\cdot} resulting from this

reaction reacts with O_2 to create NAD^+ and $O_2^{\cdot-}$ (Halliwell 1978).

The role of the $O_2^{\cdot-}$ in the plant cell walls is as a precursor for both H_2O_2 and $\cdot OH$ (hydroxyl radical). These two particles, however, seem to have opposite roles in the cell wall. H_2O_2 makes the wall less extensible, which is relevant to lignification processes and cell wall cross-linking, while $\cdot OH$ leads to cell wall loosening and is linked with polymer scission (Vreeburg and Fry 2005).

Superoxide production in plant cell walls is controlled by the developmental programme, for example, the lignification of vascular tissue in spinach hypocotyls. It can be also regulated by internal and external stimuli such as mechanical stress in potato tubers (Johnson et al. 2003). Most external stimuli have been shown to induce increased superoxide production, and pathogen and pathogen-related stimuli have been particularly widely studied. In addition, $O_2^{\cdot-}$ production in maize coleoptiles has been shown to be controlled by hormonal regulation (Schopfer *et al.* 2002). Experimentation showed that $O_2^{\cdot-}$ production in coleoptile segments was enhanced by the addition of exogenous auxin.

The life-cycle of $O_2^{\cdot-}$ production is brief because SOD is the major scavenger of $O_2^{\cdot-}$ in the apoplast. Most SOD found in cell wall is the Cu/Zn SOD, which catalyses the dismutation of $O_2^{\cdot-}$ to produce O_2 and H_2O_2 . It has also been proposed that, to dismutate the $O_2^{\cdot-}$ yielded by NADPH oxidases, SOD is localized near membrane-bound enzymes. $O_2^{\cdot-}$ can also be scavenged through reactions with phenolics, ascorbate and glutathione (Vreeburg and Fry 2005).

1.2.2.3 Detection of superoxide production

There are three main methods for detecting $O_2^{\cdot-}$. The first of these is particularly sensitive and involves the chemiluminescence of lucigenin (bis-N-methylacrodinium) that has been successfully applied to membrane vesicles. A signal is provided in this method by the production of $O_2^{\cdot-}$ that results from auto-oxidation when lucigenin is used in too high a concentration (Li *et al.* 1998). The second method is used to investigate apoplastic $O_2^{\cdot-}$ production, and involves the SOD inhibitable reduction of cytochrome c (Murphy *et al.* 1998). This method has been used for superoxide determination in both glyoxysome

(Sandalio *et al.* 1988) and leaf peroxisome (del Rio *et al.* 1989). The third method is employed to confirm apoplastic $O_2^{\cdot-}$ production, and utilizes tetrazolium salts. The latter are used because nitro blue tetrazolium (NBT) generates blue formazan deposits after reduction by $O_2^{\cdot-}$, and this change of colour can be measured by a spectrometer. The appearance of blue formazan deposits, due to the reaction of NBT with superoxide, can also indicate the areas of superoxide formation. For example, superoxide has been found in leaves undergoing photo-oxidative stress by its reduction of NBT to formazan deposits (Fryer *et al.* 2002). In this study, the tip region of the leaf, which had been exposed to strong photo oxidative stress, exhibited stronger staining than non-stress regions, with the majority of the staining being related to the mesophyll tissue. Superoxide radical production in the tissues of mechanically stressed potato tubers has also been directly measured using this NBT method (Johnson *et al.* 2003).

1.3 Superoxide dismutase (SOD)

Superoxide dismutase (SOD) is included among the oxidoreductases, one of six functional classes, by the International Union of Biochemists (I. U. B) and is the primary defence against potentially noxious reactions of $O_2^{\cdot-}$. This is because SOD catalyses the dismutation of superoxide radicals into oxygen and hydrogen peroxide. Unlike most other living organisms, plant SODs exist in multiple forms (isozymes). Baum and Scandalios (1979) first demonstrated the existence of SOD isozymes in plants and established their genetic basis. SOD is an intracellular enzyme that is found in every cell and remains comparatively stable. SODs increase when plants are under environmental stresses, while they are also known - in animals and humans- to decrease with age. A number of studies have also indicated that oxidative stress increases SOD activity in both prokaryotes and eukaryotes (Scandalios 1993).

1.3.1 Classification of SOD

There are three distinct types of SODs, which are classified according to whether the metals present at the catalytic site are copper and zinc (Cu/Zn SOD), manganese (Mn SOD), or iron (Fe SOD). These three types of SODs can be grouped into two evolutionary

families because Mn SOD and Fe SOD are related in their phylogenetic aspect, as a result of amino acid sequence homology, while Cu/Zn SOD forms an independent line (Stallings *et al.* 1984).

Cu/Zn SODs are commonly found in the cytosol of eukaryotic cells and chloroplasts and are the most abundant SOD in higher plants, with the exception of two plant species of Nymphaeaceae (Bridges and Salin 1981). As a result, most Cu/Zn SODs have been purified from the tissues of higher plants, for example spinach leaves (Asada and Kiso 1973), watermelon cotyledons (Bueno and del Rio 1992) and camellia pollen (Xiao-hong 2005). The isozymes of these have also been reported in tobacco leaves (Sheng *et al.* 2004), wheat and spinach (Lumsden and Hall 1974), and *Allium sativum* (Teng *et al.* 2003). Almost all of the Cu/Zn SOD enzymes purified so far from eukaryotic cells are homodimers, and the molecular weight of these is around 32,000 Daltons (Da), including two protein subunits.

Mn SOD is usually localized in the matrix of mitochondria and in prokaryotes, but a membrane-related Mn SOD has also been isolated from chloroplast thylakoids (Hayakawa *et al.* 1984). In pea leaves, Mn SOD is localized in mitochondria as well as peroxisome (del Rio *et al.* 2003). In higher plants, Mn SODs are mainly present in mitochondria from different plants (del Rio *et al.* 1992, Halliwell and Gutteridge 2000). On the other hand, in the fruit and leaves of most plants, Mn SOD has been shown to be only 3-5% of the total SOD activity (Fridovich 1986), although this figure can be as high as 20% in peas (Sevilla *et al.* 1980). The subunit weight of Mn SOD is around 23,000 Da and the enzyme may be dimeric or tetrameric.

Fe SODs are generally found in prokaryotes and have also been found in three families of higher plants - *Ginkgoaceae*, *Cruciferae* and *Nymphaeaceae*. Up to now, in all plant species examined, it is inferred that Fe SOD is located in chloroplasts (Alscher *et al.* 2002). As with Mn SOD, the subunit weight of Fe SOD is close to 23,000 Da and the enzyme may be a dimer or tetramer. The amino acid sequences of Fe SODs are very similar to those of Mn SODs, but quite distinct from the sequences of Cu/Zn SODs.

1.3.2 The function of SOD

Plants, because of their inability to move freely, have considerably more dedicated defence mechanisms against environmental changes than animals do. An increase in SOD activity in plants can be a form of systemic signalling and a direct stress indicator against environmental alterations which expose the plant to abiotic stresses from herbicides, drought, high and low temperatures and pollutants. A single, reliable method for SOD determination can, therefore, be very helpful when examining plant physiology.

Antioxidants, including SOD and free radicals, also play an important role during developmental and natural senescence processes in plants. In maize, for example, early senescence has been shown to result from enhanced H_2O_2 production, lipid peroxidation and lower SOD (especially Mn SOD) levels (Dagmar *et al.* 2001). SOD activity has also been demonstrated to increase with growth, development (Baum and Scandalios 1979), and the ripening of fruits (Jimenez *et al.* 2002).

1.3.3 Factors affecting SOD changes

As was mentioned above, SOD has a role in protecting leaves against photodynamic damage. In higher plants, $O_2^{\cdot -}$ is a major factor in photodynamic damage and, thus, SOD contributes to protecting against photodynamic processes. In leaves of *Impatiens flanaganiae*, there was a rapid increase in the activity of SOD and catalase when plants were exposed to relatively high light intensity (L'all and Nikolava 2003).

SODs are metalloenzymes - containing copper and zinc, manganese or iron - and so a nutritional shortage in one of these metals is thought to result in a decrease in the corresponding SOD. This may also have the effect, in the case of a cell which includes more than one type of SOD, of a nutritionally imposed reduction in one SOD resulting in compensatory increment in another SOD. For example, a manganese deficiency in peas has been shown to reduce the amount of Mn SOD, while causing a consequential increase in the level of two Cu/Zn SOD isoenzymes. In these plants, on the other hand, an increase in total SOD activity could be induced from increasing the copper supply (Rabinowitch and

Fridovich 1983).

Herbicides can also influence changes in SOD levels. These herbicides are compounds which can be reduced within cells, and whose reduced forms react swiftly with dioxygen and result in increased production of $O_2^{\cdot-}$ and H_2O_2 . Ultimately, enhanced levels of intracellular SOD offer a defence against $O_2^{\cdot-}$ concentrations resulting from the toxicity of these compounds. For example, methyl viologen (bipyridylum herbicide) has effects on the levels of chloroplastic SOD as well as mitochondrial and cytosolic SODs (Dodge 1994, Van Camp *et al.* 1994). On the other hand, transgenic tobacco plants that overexpressed SOD and APX in chloroplasts exhibited enhanced tolerance against methyl viologen-mediated oxidative stress (Kwon *et al.* 2002).

A further factor controlling SOD levels is SO_2 , a major pollutant that swiftly hydrates to sulfurous acid, which then ionizes to sulfite. Sulfite is subject to autooxidation by a free radical chain pathway, and this produces an intense oxidizing species when the reaction is induced by $O_2^{\cdot-}$ (Asada and Kiso 1973). In this context, SOD prevents the initiation of the chain oxidation of sulfite. It has been shown that the young leaves of poplars, which have a 5-fold higher SOD content than older leaves, are more resistant to SO_2 than older leaves (Tanaka and Sugahara 1980).

Thermal effects, both at high and low temperatures, can also be related to changes in $O_2^{\cdot-}$ production, which in turn causes changes in the levels of SOD. For instance, chilling tomato leaves reduces the amount of both SOD and manganese found in the plant (Kaniuga *et al.* 1979). Furthermore in wheat, differential expression of Mn SOD sequence variants occurred during cold acclimation (Baek and Skinner 2006).

A plant's reaction to drought stress is a very complex phenomenon in which abscisic acid has a major function, because it induces stomatal guard cells to close, while simultaneously decreasing water loss. This process correlates with a decrease in CO_2 for photosynthesis, which can result in the formation of ROS because of electrons being misdirected in the photosystem. Mechanisms for reducing oxidative stress are, therefore, crucial in increasing drought tolerance in plants. This has been shown by research that induced cytosolic Cu/Zn

SOD in tomatoes through intense drought, and by resistance in drought-tolerant maize being found to correlate with increased activity of both Cu/Zn SOD and glutathione reductase (Arora *et al.* 2002).

1.3.4 Studies of SOD in plants

1.3.4.1 Developmental changes and SOD activities

There have been a number of papers published in this area. In tomatoes (Perltreves and Galun 1991), it was found that the expression of the two Cu/Zn SOD genes present depended on the organs and the developmental stage of the plant and on the levels of enzyme activity. In the published research, cDNA clones were used as probes for chloroplast Cu/Zn SOD (clone T1) and cytosolic Cu/Zn SOD (clone P31). When this was performed, the two genes presented relatively different expression patterns. The T1 transcript was rare or absent in roots, stems and ripening fruits, whereas the P31 transcript was abundant. The shoot tips, seedlings, flower buds, and young leaves, however, presented high levels of the two mRNAs at all developmental stages.

In Norway spruce, the effect of developmental changes on SOD activity, and on the presence or absence of isozymes, were also studied (Kroniger *et al.* 1993). It was found that there were two Cu/Zn SODs (SOD I and SOD II) and one Mn SOD (SOD III) in the spruce spruce seeds, seedlings and foliar buds. With these SODs, the highest activities were found in the buds and germinating seeds. The major isozyme in the buds and seeds was SOD II, while SOD I was the major isozyme in mature needles. Although SOD III was present at all developmental stages of the seedlings, it disappeared during the bud break and reappeared in the mature needles. The highest SOD activities occurred in young differentiating tissues of Norway spruce, implying that younger tissues need higher protection against oxidative stress than do photosynthetically active needles. It seems that the appearance of the three SOD isozymes was independently regulated with regard to developmental stages and seasons.

In the seedlings and developing kernels of maize, the steady-state levels of cytosolic (Cu/Zn) and mitochondrial (Mn) SOD mRNAs were investigated by means of an RNA blot

analysis, which used Cu/Zn and Mn cDNA encoding these isozymes as molecular probes (White *et al.* 1990). In this experiment, the mRNA levels of SOD3 (Mn SOD) increased in postgerminative scutella, while Cu/Zn SOD levels remained at a constant level. Therefore, mRNA levels transcribed by nuclear genes encoding mitochondrial SOD seemed to be independently regulated through different developmental stages.

In transgenic tobacco, the activity, at various developmental stages, and regulation of cytosolic Cu/Zn SOD in *Nicotiana plumbaginifolia* was studied (Herouart *et al.* 1994). Transcriptional regulation at the cellular level was studied by fusing the promoter of the *Nicotiana plumbaginifolia* cytosolic gene, encoding Cu/Zn SOD(SODCc), to the β -glucuronidase (GUS) reporter gene (*gusA*), and the resulting activity was assayed. In this transgenic plant, the activity of GUS was much higher in mature flowers than it was in flower buds. Furthermore, according to an analysis of the expression of SOD Cc-*gusA* at various developmental stages of the flowers, there was very high GUS activity in the stigma regardless of the developmental stage, but only dehiscent anthers exhibited GUS activity in the stamen.

1.3.4.2 SOD in flowers and fruits

In one study in this field, *Chrysanthemum morifolium* petals were divided into five stages from stage one (blooming) to stage five (complete wilting) in order to characterize the physiological status of petals over the senescence period (Bartoli *et al.* 1995). SOD activity increased from stage one to stage three reaching the maximum value, but this was followed by a decrease in the subsequent stages. Data from this study suggested that lipid peroxidation and membrane damage are incorporated in petal senescence. The significant increase in antioxidant enzymes, including SOD, in the initial stages of senescence seemed to be triggered by control mechanisms against damage from senescence in the petals.

Subsequent research into petals of the daylily (*Heemerocallis* hybrid) showed symptoms of senescence 24 hours after flower opening, which were preceded by increases in ion leakage. This seems to result from losses of differential permeability in membranes which have been associated with ROS. Furthermore, this study suggested that membrane changes, leading to petal senescence and cell death, may be triggered by enhancing lipoxygenase

activity and ROS, due to decreases in protective enzymes such as superoxide dismutase and catalase (Panavas and and Rubinstein 1998).

Moreover, it has been found that free radicals and antioxidants have a significant role during the senescence process of petals. The closely related phenomena of ethylene biosynthesis and membrane breakdown, when they involve free radicals, are also further factors that can affect SOD levels. It is now clear that 1-amino cyclopropane-1-carboxyl acid (ACC) is the precursor of ethylene, and that oxygen is necessary for ethylene production. Hence, O_2^- and other ROS may be implicated in this process, and either SOD or catalase could inhibit ethylene production (Legge *et al.* 1982). In cut carnations, for instance, ethylene content increases with peroxidation during petal development, while the content of SOD and catalase drops between the initial stage and blooming (Sylvestre *et al.* 1989).

SOD activities during flower budding and fruit development of apple have also been studied (Abassi 1998). In the dormant flower buds of this plant, SOD and catalase activities were very low, but these then increased 2 – 5 fold during bud swelling. SOD activity fell 5 – 8 fold, however, with the onset of the bud break and during further fruit development. In fact, SOD activity was only detected in the peel, but not in the cortex and seed tissue, of immature and mature apple fruit.

Similarly, when pepper and cucumber fruits were studied, SOD activity was mainly detected in the pepper's pericarp and in the skin and peeled pericarp of the cucumber (Rabinowitch and Sklan 1981). In cucumber fruits, the level of SOD activity in the skin was 3 – 4 times higher than in the pericarp, as a result of protective mechanisms against exposure to harmful environments. In both fruits, the SODs were identified as Cu/Zn SODs from inhibitor reactions, with molecular masses of about 32,000 and 40,000 Dalton respectively. It was also found that SOD activities were high in immature-green fruits of both species, but these decreased to a minimum during the mature green stages. SOD levels went up again, however, until the peppers turned orange in colour and the cucumbers became yellow, and then only declined again after this ripening.

It has been found that free radicals have a significant role in fruit ripening, and that superoxide dismutase and catalase are the most important antioxidant enzymes influencing fruit ripening patterns. During maturation and ripening of the fruit of the *Amelanchier alnifolia* (Rogiers 1998), the ripening process was accompanied by a considerable increase in the free-radical mediated peroxidation of membrane lipids, while the activities of SOD and CAT, from the mature green to the fully ripe stage, decreased 4 fold and 18 fold respectively.

1.3.4.3 SOD in pollen

A few studies have also been conducted on SOD in pollen. In one of these, a superoxide dismutase was purified and characterized from camellia pollen (Xiao-hong *et al.* 2005). The SOD in camellia pollen was identified to be Cu/Zn SOD with a molecular weight of 69,500, and it was found to be a dimer composed of two identical subunits. The isoelectric point of this enzyme was 4.1, and the N-terminal amino acid was identified as glycine.

In olive pollen, the identification and immunolocalization of superoxide dismutase isoenzymes were studied (Alche *et al.* 1998). Crude extracts from olive tree pollen were subjected to a native PAGE gel, which showed the existence of four isozymes. All the SODs were identified as being from the Cu/Zn SODs family, and the isoelectric points for the four isoforms were 4.60, 4.78, 5.08 and 5.22. This study also identified, after SDS-PAGE and immunoblotting, a main polypeptide band of 16.5 kDa, which was consistent with the molecular masses of Cu/Zn SOD subunits that have been found in other plant sources. Furthermore, the immunocytochemical studies conducted in this research discovered that Cu/Zn SOD was concentrated in the cytoplasm of both vegetative and generative cells, and that this also adhered to the excine in the pollen grain, implying a protective function against oxidative stress during pollen development.

Another study examined Ole e 5, a pollen allergen of *Olea europaea*, which has been identified as a SOD. The cDNA of Ole e 5 was cloned, expressed in a complete form in *Escherichia coli* and characterized for immunoreactivity. Through a sequence analysis of Ole e 5 cDNA, Ole e 5 was confirmed as a Cu/Zn SOD, with an identity 80 – 90% consistent with SOD from other species (Butteroni *et al.* 2005).

Prior to all of the above research on SOD in pollen, superoxide dismutases were purified and demonstrated in extracts of anthers and pollen from *Zea mays*, and in Baxtin and Polbax - two related products (Oden *et al.* 1992). The samples were purified by adding soluble poly-N-vinylpyrrolidone, precipitating ammonium sulphate and gel filtration chromatography. After purification, the maize extracts, Baxtin and Polbax, showed similar elution patterns. Fractions with superoxide dismutase activity were then analysed on native PAGE gel and stained by NBT. The results indicated common occurrences of Cu/Zn SOD and Mn SOD in extracts of anthers and pollen of *Zea mays*, Baxtin and Polbax.

1.3.4.4 SOD in seeds

The generation of active oxygen species (AOS) is incorporated in aspects of seed physiology, such as germination and aging, and may result in oxidative stress and cellular damage. Consequently, antioxidant enzymes, including SOD and detoxifying mechanisms, have an important role in the completion of seed germination and in seed storability (Bailly 2004). Beans, for example, have been shown to exhibit this process, with a 3 – 5 fold increase in SOD activity between germination and the first few days of growth (Matkovic 1977). Similar patterns were reported, moreover, in peas and oats (Giannopolitis and Ries 1977), although there were important differences between the two species during early germination. The principal difference was that SOD specific activity in oats increased rapidly during the first days of germination, while in peas it remained at the initial level at this stage. In peas, it was found that the most significant increase in SOD specific activity occurred during greening and hook opening.

The activities of antioxidant enzymes and other antioxidants during the first stages of germination have been studied in *Chenopodium rubrum* seeds (Ducic *et al.* 2003). In this study, SOD activity was highest during radicle protrusion and seedling development, while SOD and CAT activities were highest before radicle protrusion. It was also found that concentrations of oxidized and reduced glutathione remained constant during germination, with the highest levels detected at the time of radicle protrusion. Ascorbic acid was only detected preceding radicle protrusion, whereas its oxidized form was present during the whole germination period. In general, therefore, antioxidant enzymes, such as SOD and CAT, increased significantly prior to radicle protrusion, while oxidized concentrations

diminished during further germination of these seeds.

Previously, SOD in rice at different physiological stages, and in various breeding lines, were characterized using PAGE (Pan and Yau 1991). Similar multiple SOD patterns were present at different stages during seed germination and seed development - namely, four Cu/Zn SODs and two Mn SODs with pIs, detected by isoelectrofocusing, between pH 5.2 and 5.8. The Cu/Zn SODs contributed to most of the total SOD activity at the physiological stages studied, and the SOD isozymes in the ten lines of rice examined were very similar.

In a later study, Cu/Zn SOD from the *Radix lethospermi* seed that is used as a medicinal material was purified and characterized by ammonium sulfate fractionation and successive column chromatographic procedures including DEAE-52, Sephadex G-200 and DEAE-52 again. The purified enzyme had a specific activity of 4843 U/mg, and was purified 267.2 fold with a yield of 23.55%. The molecular weight of this enzyme was about 30,500, with two non-covalently joined equal subunits. In this research, Cu/Zn SOD was found to be a novel thermostable protein and to have other properties that are very similar to Cu/Zn SODs from plant sources (Haddad and Yuan 2005).

1.3.5 SOD inhibitors

Cyanide is an extremely powerful inhibitor of Cu/Zn SODs, and H₂O₂ also inhibits these enzymes irreversibly. This is because that both cyanide and H₂O₂ interact with the Cu of the enzymes (Scandalios 1993). These Cu/Zn SOD enzymes are also inactivated by extended incubation with diethyldithiocarbamate, which binds to the copper at the active sites and consequently removes this metal from the enzyme (Halliwell and Gutteridge 1985). As a result, this susceptibility of Cu/Zn SODs to cyanide has been utilized as an important tool to distinguish Cu/Zn SODs from Fe SOD and Mn SOD, as cyanide has no effect on these latter two types of enzyme. Mn SOD is not inhibited by cyanide, H₂O₂ or diethyldithiocarbamate, but it can be removed through treatment with chloroform plus ethanol. Fe SOD is similar to Mn SOD in that it is not affected by cyanide, while it is distinguishable in that it is irreversibly inactivated by H₂O₂ (Halliwell and Gutteridge

1985).

1.4 Other antioxidants in plants

1.4.1 Catalase

Hydrogen peroxide causes the formation of $\cdot\text{OH}$ radicals, and thus it damages living organisms. Compared with other plant cells, photosynthesizing plant cells show especially high rates of H_2O_2 production. Furthermore, low concentrations of H_2O_2 can rapidly inactivate photosynthesis because of the way they inhibit CO_2 fixation. It is thought that H_2O_2 is especially harmful, even though H_2O_2 is a less reactive oxidant when compared to other ROS, because it is comparatively stable and may therefore diffuse within cells. As a result, H_2O_2 can generate more ROS, which greatly increases its toxicity, meaning that the effective scavenging of H_2O_2 is very important for the survival of plants. This indicates, in turn, that the fact that hydrogen peroxide can be converted to oxygen and water by the enzyme catalase means that this enzyme plays an important role in plants. Generally catalases are small subunit monofunctional enzymes, or tetrameric proteins with a molecular weight of around 240 kDa. The typical inhibitors of these plant catalases are cyanide, azide and 2-aminotriazole. In addition, micromolar concentrations of nitrate and millimolar concentrations of sulfite can also inhibit plant catalases (Streb *et al.* 1993).

1.4.2 Ascorbic acid and ascorbate peroxidase

Ascorbate is both the most abundant small molecular weight antioxidant and the most important antioxidant in plants. Its basic role is the elimination of hydrogen peroxide. High concentrations of ascorbate are commonly found in fruits, but ascorbate concentrations in fruit are, in fact, not always higher than those found in leaves and may even be lower in some species (Davey *et al.* 2000). There are two successive steps in the oxidation of this ascorbate: firstly, mono-dehydro-ascorbate is generated, and secondly, the mono-dehydro-ascorbate is converted disproportionately to ascorbate, or dehydro-ascorbate if it is not swiftly re-reduced to ascorbate (Foyer 1993). In addition, ascorbate peroxidase,

participating in the removal of H_2O_2 , is found in almost every compartment of the plant cell. Its activity has been frequently reported in the chloroplasts and cytosol, but some recent research has shown that it is also active in mitochondria (Anderson *et al.* 1995).

1.4.3 Glutathione and glutathione reductase

Glutathione and glutamyl cysteinyl glycine (GSH) react biochemically with a variety of active oxygen species (AOS). GSH has been linked to the detoxification of H_2O_2 in the ascorbate-glutathione cycle through enzyme-catalysed reactions (Noctor and Foyer 1998). GSH also contributes to ascorbate regeneration by way of dehydro-ascorbate reductase, and it is also formed by glutathione reductase (GR) in a NADPH-dependent reaction.

1.4.4 α -Tocopherol and carotenoids

α -Tocopherol, a membrane-associated antioxidant, is a scavenger of lipid peroxide, $\cdot\text{OH}$ and $\text{O}_2\cdot^-$, and can prevent lipid peroxidation. Carotenoids can scavenge $\cdot\text{OH}$, $\text{O}_2\cdot^-$ and peroxy radicals, and can also prevent the oxidation of vitamin A. Additional functions of carotenoids are to quench singlet oxygen and to shield cells by absorbing excess excitation energy from chlorophyll (Arora *et al.* 2002).

1.5 Introduction to Petunias

The plant with the common name of 'Petunia' (scientific name: *Petunia hybrida*) is, throughout the world, a widely-cultivated genus of flowering plants because of its wide range of colours and sizes, and because of its variety and versatility. Petunias, as tender perennials, can grow well in all zones except for very cold areas, and they are greatly favoured for summer flowerbeds, greenhouses and window boxes. As a result, this plant has been continuously bred, particularly over the past 30 – 40 years, to the point where there are now approximately 400 – 500 cultivars available (Kessler 1999).

1.5.1 Origin, history and spread of petunias

The name ‘Petunia’ is a Latinized version of the aboriginal South American name for tobacco, *petyn*, which is a close relative. The ancestors of most petunias now in cultivation are thought to be *Petunia axillaries* and *Petunia violacea*, both from Argentina (Shosteck 1974). Early breeding work by a French botanist resulted in number of different varieties appearing in private gardens from around 1850. Subsequently, the forms that became known as ‘Superbissima’ were bred by Theodosia Shepherd in California in 1880, and then the multiflora and grandiflora varieties appeared from open pollination in 1940. The first F₁ hybrids were grown in 1950. Nowadays, the grandiflora singles (the term ‘single’ denotes flowers on a stem with one sepal per flower) are the most popular, followed by the multiflora singles, and the double (multi-petalled flowers on a stem with two sepals per flower) flower types. Recently, floribundas, produced as hybrids between single multifloras and grandifloras, have also become popular (Kessler 1999).

1.5.2 Classification

Petunias belong to the division ‘Magnoliophyta’, the class ‘Magnoliopsida’, the order ‘Solanales’, and the family ‘Solanaceae’ which includes 90 genera. The genus *Petunia* is extremely diverse as a result of hybridization and propagation, but it can be grouped into four major classes by considering origins, morphological characteristics and plant growth habits (Kessler 1999).

The first of these classes, the ‘grandiflora’, appeared in early 1950 and the first F₁ hybrid grandiflora, ‘Ballerina’, was produced in 1952. Grandifloras have a few large flowers, 8.5 to 12 cm in diameter, and their cultivars have been developed in a variety of colours and with petals which have frilled or rounded edges. Recently, new cultivars, with different coloured veins in the petals, have been developed and grandiflora doubles, which look like carnation flowers because of their multi-petalled shape, have also appeared. Most modern grandifloras tend to have more compact flowers in comparison to older variety. In general, grandifloras are the most popular type today, but they usually have to be kept in containers because of their susceptibility to adverse garden conditions. The second class, the

‘multiflora’ petunia, first appeared in late 1940, and ‘Comanche’, the first F₁ hybrid multiflora, was developed in 1953. Compared with the grandifloras, multifloras have more abundant but smaller flowers (3.5 to 5 cm in diameter), and have an advantage in that they have greater survivability in adverse weather situations. Multifloras, at one time, were not as popular as grandifloras, because of their small flower size, but they are swiftly gaining popularity in the southern United States as a result of careful breeding that is producing a more varied range of colours. The third, or ‘floribunda’, type of petunia was developed recently through the hybridization of grandifloras and multifloras. Floribundas can be difficult to distinguish from the above two classes, as their common characteristics are somewhat in between those of grandifloras and multifloras in regard to flower number, size and growth habit. Their primary distinguishing characteristic, and their value, is that they have improved disease resistance. The last type, the ‘milliflora’, is a true miniature plant as a consequence of a genetic mutation from *Petunia × hybrida*. This type is also popular because it is suitable for small hanging baskets, small pots, containers and window displays.

The multiflora ‘Hurrah’ variety, chosen for this study, is similar to the grandifloras in terms of its morphological characteristics and production timing, and it shows its flowers sooner than other multifloras. Hurrah is, however, regarded as more attractive than most common grandifloras in that it continues to bloom in heat and humidity, and has more colour choices and more compact flowers compared to most – and particularly the older - varieties of grandifloras.

1.5.3 Favourable characteristics for this study

Petunias form self-branching mounds, with a height of 0.3 to 0.6 m. Their leaves, 10 to 12 cm in length, are ovate to ovate-lanceolar in shape and their seeds are very fine. The flowers are trumpet-shaped and show either singly or doubly. Flower diameters are 5 to 12 cm and come in a variety of patterns (edged, striped, or starred) with contrasting colours. The flowers of ‘Hurrah’, used in this research, are single-petalled in form (3.5 to 7.5 cm across), white in colour, of a simple trumpet shape, and usually have one pistil, five stamens and one sepal per flower.

Petunia flowers are particularly advantageous as a study material because they do not show any diurnal rhythm in the opening of their flowers (Franzmayr 1999). Therefore, it is possible to collect flowers for experimentation at any time of day without concerns about biochemical and physiological changes. Petunias also grow well in all but the coldest zones, and consequently they are easy to cultivate for study in a growth room or glasshouse. A further advantage for researchers is that they have a short life cycle and can begin flowering within a few months of being sown. They also have a habit of progressive flowering, which offers continuous blooms and produces large numbers of flowers throughout their lifetimes. As petunias are quantitative long-day plants, higher quality of flowering and successive blooms can be triggered simply by slight increases in temperature (night temperature 1 – 3 °C, day temperature 6 – 10 °C), longer photoperiods (>13 hours) and supplemental light in the glasshouse. Furthermore, Petunias are almost as widely planted as roses, both for garden displays and for the production of cut flowers, throughout the world. Therefore, the study of beneficial enzymes in these common plants may facilitate easy and direct applications in industry and medicine.

1.5.4 Studies of enzyme activities in Petunias

There has been some research that has already attempted to identify enzyme activities in petunias. For example, there has been a study into invertase activity in petunia petals (Allen 1993), a study of β -1,3-glucanases within healthy petunia flowers (Price 1997) and a study of flavanone-7-O-glycosyltransferase activity from *Petunia hybrida* (Durren *et al.* 1999). There has been, however, only one study of superoxide dismutase in petunias, and this examined the ‘cloning and nucleotide sequence of a petunia gene encoding a chloroplast-localized superoxide dismutase’ (Tepperman *et al.* 1988). To date there has not been any publication on the characteristics and purification of superoxide dismutase activity in petunia flowers. This study, therefore, is focused on superoxide dismutase activities and purification in the male part of petunia flowers. This is particularly relevant because this is one of the most important enzymes in terms of human health.

1.6 Applications of SOD

Anti-oxidative stress mechanisms, including SOD, in the human body are limited to the inside of cells and to tissues where ROS occur. If the quantity of ROS exceeds the ability of the defence mechanisms of the body to respond, severe diseases, such as cancer and arteriosclerosis, may develop (Ukeda 2004). Moreover, cell damage as a result of excessive ROS is also considered to be one of the principal causes of aging and aging-related diseases. Therefore, there has been much interest in the use of ROS trapping agents for their potential use as anti-aging agents and as a possible cure for many diseases. To this end, SODs are often included in nutritional supplements, and as coenzymes in cosmetic products, in order to combat anti-oxidative stress and to prevent aging. More specifically, SOD is included in a variety of cosmetic products to decrease free radical damage to skin, because SOD is found naturally in both the dermis and epidermis and is crucial for the production of healthy fibroblasts. SOD is also used in decreasing fibrosis following radiation for breast cancer (Campana 2004). As a result of all of these interests, SOD can nowadays be applied in a variety of forms - including injections, sublingual oral supplements, enteric-coated pills, and topical creams. In its topical form, for example, it is thought that SOD will help to decrease scar tissue, heal wounds and protect against harmful UV rays (Paramonov *et al.* 2005).

SODs are also applied as treatments for some diseases because of their function as anti-inflammatory and autoimmune agents. In these cases, SODs are applied to compensate for a lack of natural SOD, and to help neutralize free radicals, in patients with Crohn's disease (Phylactos *et al.* 2001), prostate problems, corneal ulcers, inflammatory diseases and rheumatoid arthritis (Eugenia *et al.* 2005). Furthermore, the recent significant developments in SOD therapy in two new areas, namely ischaemia-reperfusion and the practice of grafting and transplantation (Domanski *et al.* 2006), suggest further uses for the SODs.

Determination of SOD activity could also be employed as a means of indicating and diagnosing certain health conditions, as well as for investigating the mechanisms that cause disease, because the correlation between some diseases and SOD activity appears to be

direct and strong. For instance, the SOD activity level in diabetes patients is very low, as SOD activity is dramatically reduced by the *in vivo* Maillard reaction, and, moreover, continuous decreases in SOD level points towards the development of diabetes complications (Ukeda 2004). In addition, the detection of increased levels of SOD-1 in amniotic fluid is now used as a screening test for Down's syndrome in the foetus. Higher levels of SOD-1 can also be detected in the blood, sera, and other extra-cellular body fluids of a mother carrying foetal Down syndrome (Netto *et al.* 2004).

As interest has increased in the anti-oxidative qualities of plants, the study of SOD has come to play an important role in the genetic engineering of crops. There have, for example, been a number of studies into the genetic variability of plants that contain SOD – including research that found that the gel pattern of SOD isozymes was affected by both the iron supply and the age of plants (Garcia *et al.* 1981). This research into the effects on SOD isozyme production of various environmental alterations has, in turn, predicted the possibility of research into the sophisticated genetic control of many plants. Recently, for instance, genetic engineering for stress tolerance in crop and forest plants has been a high research priority in plant biotechnology, and a number of transgenic plants with controlled SOD levels have been introduced. An example of this was the creation of transgenic alfalfa plants that overexpress either Mn SOD or Fe SOD cDNA (McKersie *et al.* 1997). These transgenic plants showed 25% higher freezing tolerance than non-transgenic plants. Another study found that Mn SOD, overexpressed in *Arabidopsis thaliana*, had a significant role in protecting cells against ROS due to salt stress, and, as a consequence, this research resulted in enhanced salt-tolerance in transgenic plants (Wang *et al.* 2004). More recently, transgenic rice plants, where a Mn SOD gene from peas was introduced into chloroplasts of rice using *Agrobacterium*-mediated transformation, have presented enhanced drought tolerance (Wang *et al.* 2005). This method had also been used in an earlier study, in which Cu/Zn SOD cDNA (mSOD1) from cassava was inserted into cucumber fruits with *Agrobacterium*-mediated transformation, utilizing an ascorbate oxidase promoter, in order to create transgenic cucumber fruits containing high levels of SOD for an anti-aging cosmetic material (Lee *et al.* 2003). In this research, the transgenic fruits had levels of specific SOD activity roughly three times higher than those in non-transgenic plants.

1.7 Objectives of this study

Despite the numerous studies that have been conducted on superoxide dismutase activity in micro-organisms, animals, and the leaves, seeds, roots and shoots of plants, there has been only superficial research on SODs in flowers. Furthermore, while pollen and SOD have much in common in terms of their anti-inflammatory effects, few studies have been reported on SOD in pollen. This omission is particularly glaring as the reproductive parts of flowers could be expected to have higher SOD activity because of the need to protect genetic information against oxidative stress. Therefore, SOD activity in the male reproductive parts of the flower, including the pollen, will be the focus of this investigation.

Petunias are one of the most popular flowering annuals for display purposes in both New Zealand and, with the exception of the very coldest areas, throughout the world. Some studies into both the activities of other enzymes and on senescence have been carried out in petunias, but thus far there has been no literature that focuses specifically on superoxide dismutase activity in the petunia flower. This study is, therefore, intended to fill this gap in the research. If the male reproductive parts, including the pollen, in petunias have superoxide dismutase activity, these could be introduced as a new source for nutritional supplements, cosmetic products, and anti-aging and anti-inflammatory agents.

The objectives of this study are:

1. To investigate the occurrence of superoxide at five developmental stages of the male reproductive part of the petunia 'Hurrah'.
2. To assay superoxide dismutase activity and analyze its change during the five developmental stages.
3. To identify the superoxide dismutase isozymes that are present in the male reproductive tissues of the petunia 'Hurrah' using a native PAGE system.
4. To partially purify the enzyme, and compare the characteristics of SOD activity in the crude extracts and the partially purified enzyme preparation from the male reproductive tissues of the petunia 'Hurrah' using a native PAGE system.

CHAPTER 2

MATERIALS AND METHODS

2.1 Plant Material

Petunia hybrida variety ‘Hurrah’, which is a perennial petunia, was chosen for this research. The cuttings of this plant were grown in a potting mixture (containing 63% tree bark, 20% peat, 8.5% sterilized soil and 8.5% sand with 270 - day slow release fertilizer) and the resulting cuttings were transplanted into individual pots where they remained until they reached maturity. The plants were kept inside a glass-house maintained at 25 - 30 °C/15 - 22 °C day/night during summer and 15 - 24 °C /10 - 18 °C day/night during winter and water was provided at regular intervals to prevent the soil from drying out (Plate 1).

Flowers at five different stages of development were collected for investigation. Flowers at each of the five different stages of development (Plates 2 and 3) were collected at approximately 10:00 - 12:00 a.m for experiments such as superoxide determination and SOD (superoxide dismutase) assay. The procedure for extraction from the flowers involved grasping the filament of the stamen with forceps and then pulling the stamen free of the flower. Separation between the flower and the stamen generally occurred at the junction of the petals. The anthers and filaments were then excised with a scalpel and were used immediately for some of the experiments, for example comparing SOD activity at the five different stages of development. For the purposes of enzyme purification, however, three different groups of 1,000 stamens from different pots and days were harvested and stored in a –80 °C freezer until use.



**Plate 1: Petunia 'Hurrah' in a glasshouse
at the University of Canterbury, NZ**

2.2 Superoxide production

2.2.1 Superoxide in anthers and filaments

The presence of superoxide in the stamen was detected based on the SOD assay described by Beyer and Fridovich (1987). When the yellow NBT reacts with a superoxide, a dark blue insoluble formazan compound is produced. This is because superoxide is a major oxidant species responsible for reducing NBT (nitroblue tetrazolium) to formazan (Maly *et al.* 1989). To this end, anthers and filaments from 5 flowers in five different stages were soaked separately in 2 ml NBT solution (4.1 mg NBT in 50 ml distilled water) for up to 24 hours at room temperature and the colour change was examined with the aid of a microscope.

2.2.2 Superoxide in pollen

Superoxide production of the pollen at the five developmental stages was also determined. The predehiscent anthers of five flowers from stage 1 to stage 4 were cut in half and the pollen was scraped off, while dehiscent pollen from stage 5 was stirred in 1 ml NBT solution and incubated at room temperature for 24 hours. The total pollen number and stained pollen number present in the blue colour was counted under a light microscope (magnified 100 ×) using a haemocytometer.

Superoxide production during pollen germination was also investigated. Dehiscent pollen at the fifth stage from 5 flowers were spread on a germination medium. The latter consisted of 10% (w/v) sucrose + 1% (w/v) agar in a Petri dish and this was kept in a dark room for 24 hours. After pollen germination was confirmed using a stereo microscope, the germinated pollen was scraped gently from the surface of medium and then stirred in 2 ml NBT solution, and was incubated for 24 hours at room temperature before the colour change of the germinated pollen was observed.

A further experiment involved examining change in superoxide production during a

storage period. Dehiscent pollen harvested from 20 flowers at the fifth stage was stored in glass tubes at room temperature for 0, 3 or 10 days. After these storage periods, 2 ml NBT solution was added to the pollen and then incubated overnight. Any difference or change in colour was noted.

Finally, the effect of heat on superoxide production was also investigated. Dehiscent pollen was harvested from 20 flowers at the fifth stage and was heated separately for 30, 60 or 90 seconds in a microwave oven (high heat). After this, 2 ml NBT solution was added and incubated overnight, and any difference or change in colour was recorded.

2.2.3 Control for NBT reactions

MnCl₂, as a reagent that performs the same function as superoxide dismutase but contains no enzyme, was used for the purpose of establishing a control for the NBT reactions resulting from superoxide (Rodrigues *et al.* 2004). Anthers, filaments or pollen from stage five were put into 2 ml solution (4.1 mg NBT and 62.925 mg MnCl₂ dissolved in 50 ml distilled water). This was compared to the plant tissue incubated in appropriate NBT solution without MnCl₂.

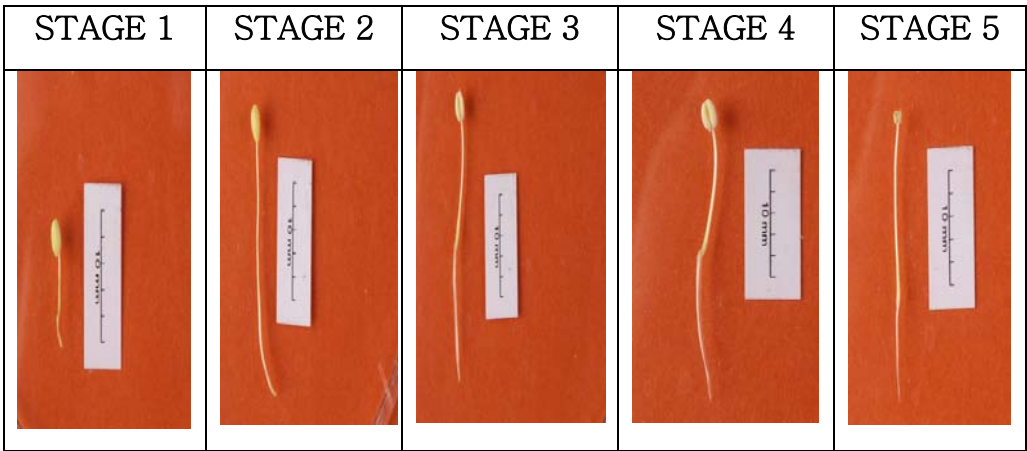
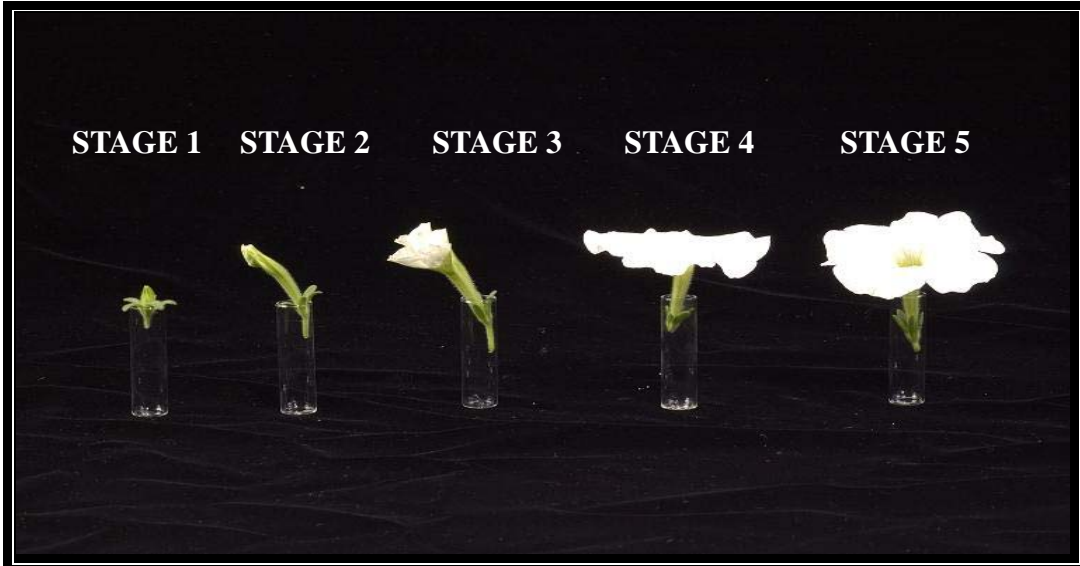


Plate 2: Five developmental stages of petunia

Stage 1: flower bud, total length 15 - 20 mm, stamen 8 - 10 mm,
tip of petals just beginning to extend, 1 days.

Stage 2: corolla extending, total length 30 - 40 mm, stamen 15 - 20 mm,
the length of corolla is roughly half of the total length, 3 days.

Stage 3: corolla fully extended, total length 55 - 65 mm, stamen 18 - 25 mm,
before petals begin to open, 5 days.

Stage 4: petals fully open but anthers predehiscent,
total length 45 - 60 mm, stamen 25 - 30 mm,
The diameter of open petals is approximately 60 - 75 mm,
petals are at an angle of 90° to the floral stem axis, 6 days.

Stage 5: petals fully open and anthers dehiscent, total length 45 - 60 mm,
stamen 25 - 30 mm, the diameter of open petals is approximately
60 - 75 mm, petals are at an angle of 90° to the floral stem axis, 7 days.



**Plate 3: A comparison of predehiscent anthers (stage 4) and
dehiscent anthers (stage 5)**

2.3 SOD activity at 5 developmental stages

2.3.1 Enzyme extraction

The tissues (stamens, anthers and filaments) were thoroughly ground in a cold mortar and pestle while adding an extraction buffer (see Appendix-A 1.1.1) on crushed ice until no fibrous residue was left. The homogenate was then centrifuged at $10,000 \times g$ at $4\text{ }^{\circ}\text{C}$ for 10 minutes. The precipitate was discarded and the supernatant, hereafter referred to as crude SOD extract, was used for the SOD assay, non-denaturing polyacrylamide electrophoresis and enzyme purification. To prepare the pollen extract, anthers from stages 1 to 4 were cut in half and scraped in 2 ml of extract buffer, while dehiscent pollen of the fifth stage was stirred in 2 ml of extract buffer. These mixtures were then centrifuged at $10,000 \times g$ at $4\text{ }^{\circ}\text{C}$ for 10 minutes and the resulting supernatant was used for analysis.

2.3.2 Preliminary experiments

SOD activity was measured in the crude extracts of tissues from 5 different developmental stages. Preliminary experiments were carried out to find a suitable extract dilution for the SOD assay. In addition, the effect of three SOD assay parameters on total SOD activity and % inhibition were studied: increasing volumes of extract (10, 20 or 30 μl), different dilutions of each volume of extract and other SOD assay reagents (e.g. EDTA). The appropriate dilution was chosen when the increase of total activity showed linear increment under 50% inhibition (Table1).

Table 1: The dilution of crude extracts with extraction buffer for SOD assay in different tissues of five developmental stages

	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
stamen	1:15	1:20	1:15	1:8	1:5
anther	1:20	1:20	1:15	1:12	1:5
filament	1:1.5	1:2	1:3	1:3	1:2
pollen	1:1	1:3	1:3	1:3	1:2

2.3.3 SOD activity assay

Crude enzyme extracts of different tissues from five developmental stages were assayed for SOD activity. The SOD assay was based on a modified form of the assay procedure described by Beauchamp and Fridovich (1971). The reaction mixture contained 1315 μ l of 0.1M potassium phosphate buffer (pH7.8), 100 μ l of 10 mM EDTA, 25 μ l of 0.13 mM riboflavin, and 10 μ l of enzyme extract or boiled enzyme extract and 50 μ l of 0.63 mM NBT were pipetted into a series of tubes. The mixture was vortexed and then illuminated for 15 min in glass test tubes (diameter : height =1 cm : 5 cm) that had been selected for uniform thickness and colour. The initial rate of the reaction was determined as the difference between the mixture with enzyme extract and the mixture with boiled enzyme extract. The absorbance of all reaction mixtures were read at 560 nm (BIO-RAD Smarspect Plus). The assay was run in triplicates and the mean values were obtained. One unit of SOD was defined as the amount that inhibited NBT photoreduction by 50% and thus the enzyme was quantified on the basis of the percentage inhibition it caused.

2.3.4 Change in SOD activity during storage period

2.3.4.1 Change in SOD activity in crude extracts

Three groups of crude extracts from the fifth stage stamens (from 20 flowers) were stored

at 4 °C and –20 °C for two days, one, two and four weeks. After each of these periods, changes in SOD activity were assayed as described in 2.3.3.

2.3.4.2 Change in SOD activity of dehiscent anthers

Dehiscent anthers at the fifth stage of development (from 15 flowers) were stored separately as three groups (each from 15 flowers) in Petri dishes at 4 °C and –20 °C for two days, one, two and four weeks. These were extracted and assayed for SOD activity as described in 2.3.3.

2.3.5 Effect of pH on SOD activity and stability

2.3.5.1 Effect of pH in the enzyme assay mixture

Crude extracts from the fifth stage stamens were diluted (1:5 from 15 flowers) with an extraction buffer (pH 7.8 potassium phosphate) and then assayed with different (pH 3.0 - pH 10.0) buffers (see Appendix-A 1.1). Citrate phosphate buffers were used at pH between 3.0 and pH 7.0, and 0.1M potassium phosphate buffer was used when the pH was 7.8. At pH of more than 8.8, a mixture of 25 mM acetic acid (12.5 ml), 25 mM MES (12.5 ml) and 50 mM Tris base (25 ml) was used and the desired pH was adjusted with either 1 M HCl or 1 M KOH.

2.3.5.2 Effect of pH on stability

Crude extracts from the fifth stage stamens were diluted (1:5) with buffers at different pH values. The effect of pH on the stability of the crude extracts was examined by performing SOD assay after different times from the diluted crude extracts (fresh, after 2 hours and after 24 hours).

2.4 Non – denaturing Polyacrylamide

Gel Electrophoresis (Native PAGE)

2.4.1 Native gels and sample preparation

All native PAGE gels were prepared and electrophoresed with the BIO RAD Mini-PROTEAN[®] II Dual Slab Cell System at a constant voltage of 200 volts for 42 minutes according to the manufacturer's instructions (see Appendix B). In all gels the concentration of acrylamide was 12% and the enzyme extract was mixed with 60% (w/v) sucrose (2:1, v/v) before loading. The crude extracts were diluted with the extraction buffer at levels that varied depending on the tissues concerned. These levels were 1:2 (v/v) for the stamens, 1:5 for the anthers and 1:1 for the filaments and pollen.

2.4.2 Staining for SOD isozymes

Detection of SOD isozymes after non-denaturing gel electrophoresis was based on the method of Beauchamps and Fridovich (1971). After the native gel electrophoresis, each gel was incubated in 20 ml of NBT solution (4.1 mg NBT in 50 ml distilled water) for 20 minutes. Then, the gel was incubated overnight in a mixture 12 ml consisting of 10 mM EDTA (800 µl), 0.13 mM riboflavin (200 µl) and 11 ml of 0.1 M potassium phosphate buffer (pH 7.8).

2.5 Protein Determination

2.5.1 Bradford Assay

This method was based on the procedure first reported by Bradford (1976), as this is a rapid and reliable dye-based assay for determining protein content in a solution. Using this method, a protein standard curve can be obtained using bovine serum albumin (BDH). Crude extract (100 µl) was vortexed with 1 ml Bradford reagent (see Appendix-A 1.2) and

left at room temperature for 10 minutes before the absorbance was read at 595nm using a spectrophotometer (BIO-RAD Smarspect Plus). The result was analyzed using an equation derived from a standard curve (Microsoft Excel).

2.6 Isolation and Purification of SOD

2.6.1 Preliminary experiment

The ammonium sulphate method was optimized in two different ways. The first was to remove unwanted proteins by a three-stage process. Initially, 0 to 30% saturation with ammonium sulphate was used, and after centrifugation at $10,000 \times g$ for 20 minutes, a further 30 to 60% saturation of ammonium sulphate was added into the supernatant. After further centrifuging, 60 to 90% saturation was added into the supernatant. The supernatants and precipitates were assayed for SOD activity at each of the three stages and specific enzyme activities were calculated and compared. Specific enzyme activity was defined as the units of enzyme activity per milligram of protein. At each step, the precipitate after centrifuging was dissolved in 10 ml of extraction buffer and then centrifuged again and used for SOD assay.

The second method of optimization was attempted to achieve the most desirable ratio directly by removing unwanted proteins in one step. Saturations of 30%, 40%, 50%, 60%, 70%, 80% and 90% ammonium sulphate were used separately after centrifuging. The precipitates were dissolved in 10 ml of extraction buffer and then SOD activity and protein content were determined in both the supernatants and precipitates.

2.6.2 Sample preparation and ammonium sulphate fractionation

One thousand stamens were homogenized with 20 ml extraction buffer and centrifuged at 4°C at $10,000 \times g$ for 20 minutes. After centrifuging, the pellet was discarded while 2 ml of supernatant was stored at -20°C for SOD activity and protein determination. Solid ammonium sulphate was added to the rest of the supernatant to 40% saturation and the

mixture was stirred together for approximately two hours in an ice bath and then centrifuged again. Following this, the supernatant was transferred to a dialysis tubing (from Medicell International Ltd, Germany) and dialysed against one L of 0.1 M potassium phosphate buffer (pH 7.8) for 24 hours with the buffer being changed every three hours. Dialysed samples were then concentrated to 3 ml by covering with powder polyethylene glycol (PEG) 20,000 (BDH Ltd), and centrifuged at 4 °C at $10,000 \times g$ for 10 minutes.

2.6.3 Ion exchange column preparation

Ten g DEAE cellulose (Sigma, St. Louis, USA) were incubated with 100 ml buffer for 2 - 3 days at room temperature. The buffer was 0.01 M potassium phosphate buffer (pH 7.8) containing (w/v) 0.02% sodium azide. This buffer was changed several times during swelling of the DEAE cellulose, and then degassed for more than 24 hours to remove air bubbles. A small amount of buffer was added to the column, the column outlet was opened to allow some of the buffer to pass, and then the outlet was closed to remove air from the dead space at the bottom of the column. The DEAE cellulose suspension was packed into a column of 1.5×25 cm and then equilibrated with five times the bed volume of the column.

2.6.4 Enzyme Purification

The column was drained until the buffer reached the surface of the DEAE cellulose. The concentrated sample (enzyme preparation from the ammonium sulphate step) was loaded into the closed column, which was washed with 100 ml of the same buffer (0.01 M pH 7.8 potassium phosphate). Forty fractions were collected at a flow rate 1.1 ml min^{-1} . After this any bound proteins were eluted with a NaCl gradient solution. To this end, 50 ml lower ionic strength buffer (0.05 M NaCl) was put into the gradient maker on the side of the gradient maker. Another 50 ml of higher ionic strength buffer (0.5 M NaCl) was put into the other side. By activating a fraction collector (BIO-RAD Model 2110) and pump controller, 40 fractions were collected at a flow rate of 1.1 ml min^{-1} . All fractions, 2.2 ml each, were stored at -20°C before protein and SOD activity determination.

2.6.5 Protein concentrations and SOD activity in the fractions

Protein concentrations in the 80 fractions from the column were read from the absorbance value at 280 nm. All fractions were investigated for SOD activity and protein determination (Bradford assay), and specific enzyme activity was calculated.

2.6.6 SDS-PAGE of proteins in the fractions

SDS-PAGE was run according to the method of Laemmli (1970), using a BioRad mini vertical slab gel system according to the manufacturer's instructions (see Appendix C). Samples were mixed with SDS reducing buffer, at a ratio 4:1 (v/v), and solubilized at 100 °C for five minutes. The molecular weight markers were phosphorylase B (108,000 Da), bovine serum albumine (90,000 Da), ovalbumin (50,000 Da), carbonic anhydrase (35,500 Da), soybean trypsin inhibitor (28,600 Da), and lysozyme (21,200 Da). Electrophoresis of the 12% gels was performed at 200 volts for 45 minutes. Then the gel was incubated in a stain solution for 30 minutes at room temperature. The stain solution was mixed with 50% (v/v) methanol, 10% (v/v) glacial acetic acid (BDH) and 0.1% (w/v) Coomassive Blue R 250 (Sigma). After staining, the gel was destained at room temperature in the solution including 10% (v/v) methanol and 10% (v/v) glacial acetic acid until protein bands were visualized.

2.6.7 Effect of temperature and pH on SOD activity

2.6.7.1 Effect of temperature and storage period

Samples of the fractions with the highest protein concentration were stored at both 4 °C and –20 °C for one, two and four weeks and then assayed for SOD activity as described in 2.3.3.

2.6.7.2 Effect of pH on SOD activity

The effect of pH in the enzyme assay mixture on the activity of the partially purified SOD was investigated. A bound fraction from the DEAE cellulose column was diluted with the extraction buffer (pH 7.8) and assayed at different pHs value as described in 2.3.5.1. The effect of pH on the stability of the partially purified SOD was examined by diluting

aliquots of the fraction with buffers at different pHs and then incubated for 0, 2, and 24 hours before SOD assays were performed as described in 2.3.5.2.

2.6.8 Detections of different SOD isoforms

2.6.8.1 Hydrogen peroxide or potassium cyanide treatment

After non-denaturing gel electrophoresis, a native gel was placed in 50 ml of 5 mM hydrogen peroxide or potassium cyanide (0.01 g dissolved in 50 ml distilled water) and incubated for 30 minutes at room temperature. Then, the gel was transferred to another Petri dish and washed with distilled water for 5 minutes. After this, detection of SOD isozyme followed the same procedure as described in 2.4.2.

2.7 Experimental design, statistics and data analysis

Experiments were designed with three replicates and repeated at least twice. Quantitative data of three different groups from the spectrophotometry assays were graphed with mean values \pm SE (Standard Error) calculated using the Microsoft Excel computer programme and standard statistical analysis. ANOVA (one way analysis of variance) and mean values were subjected to Tukey HSD All-Pairwise Comparisons tests with a significance level of $P < 0.05$ using Statistix Version 8.

SDS-PAGE gels were analyzed by measuring the distances the molecular weight standards (Bio Rad, pre-stained low molecular weight protein standards) migrated on the gels. The molecular weight of each band was calculated by using the appropriate $y=ax^{-b}$ type equation.

CHAPTER 3

EXPERIMENTS AND RESULTS

3.1 Production of superoxide

3.1.1 Superoxide production in anther and filament

The appearance of blue formazan deposits, resulting from the reaction of NBT (nitroblue tetrazolium) with superoxide, confirmed the localization of superoxide production in the male reproductive parts of the petunia flower. When the anthers from the five developmental stages were soaked in NBT solution for 24 hours, the reactions varied greatly between some of the developmental stages (Plate 4). The anthers of stages one and two were stained both very strongly and ubiquitously with a blue colour, while those of stages three and four only showed a blue colour on part of the vascular bundle. It was also noticeable that there was an absence of blue colour on the anthers of stage five. A closer examination involved observing a cross section of an anther under a light microscope (10×10). The anther from stage one presented a strong blue colour on some of its tissues, particularly the vascular bundle, while that of stage five hardly showed any formazan deposits on its vascular bundle (Plate 5).

In the filaments, the reaction with NBT was very strong at stage one, which showed an intensive blue colour (except for the part connected to the petal), while in that of stage five only a few regions of the filament showed weak NBT reaction. The filaments from stages two to four displayed heavy and patchy blue formazan deposits (Plate 6). However, it became clear that the part of the filament without any blue colouring also showed blue some formazan deposits when examined under a microscope (10×10, longitudinal section). In other words, the whole of the filament except for the end (the part connected to the petal) had blue formazan deposits particularly in the vascular bundles (Plate 7).

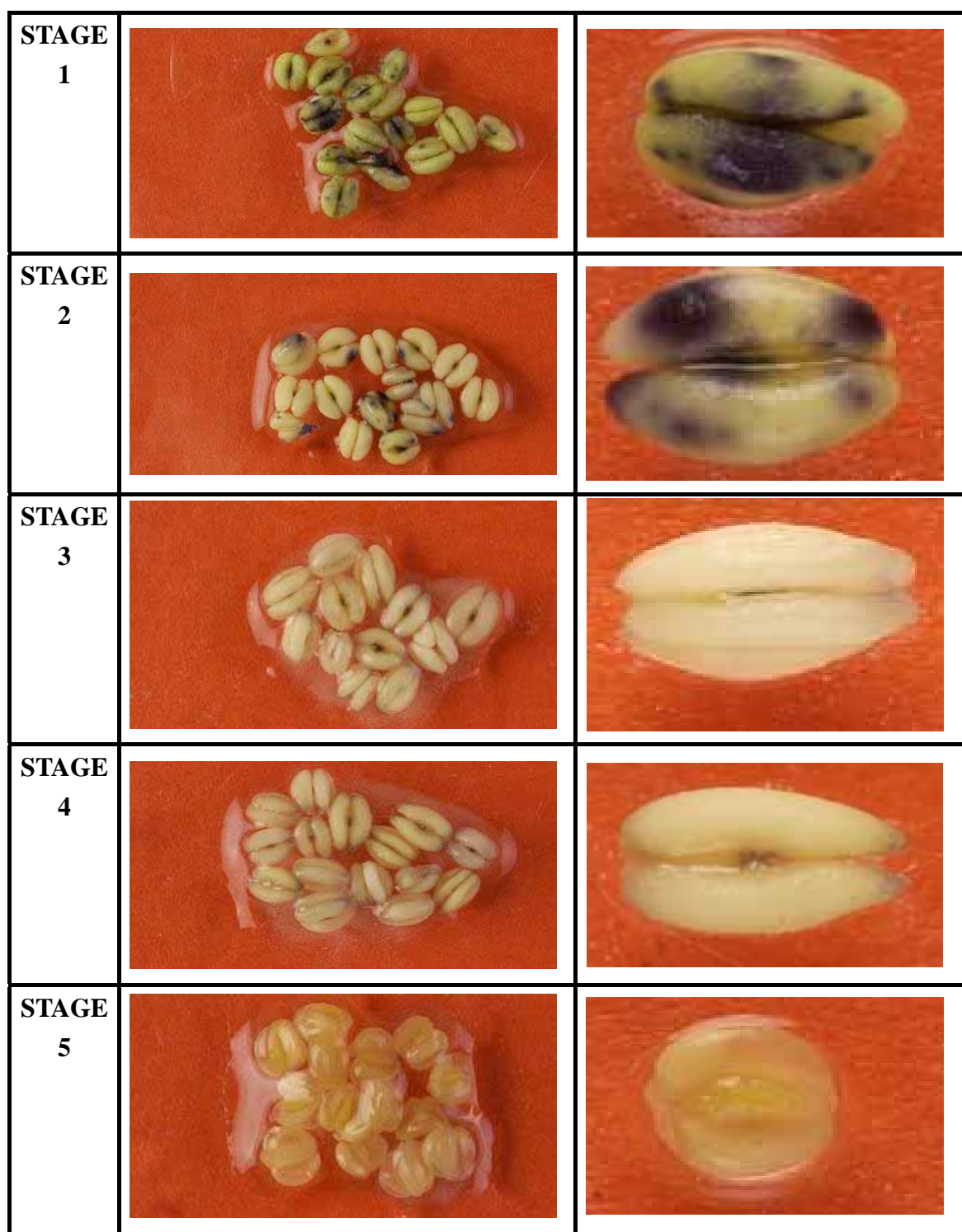


Plate 4: The reaction with NBT solution of anthers at five developmental stages

The blue colouration indicates the formation of insoluble formazan deposits that were produced when NBT reacted with superoxide.

(Length of anthers : 2.5 - 3.5 mm)

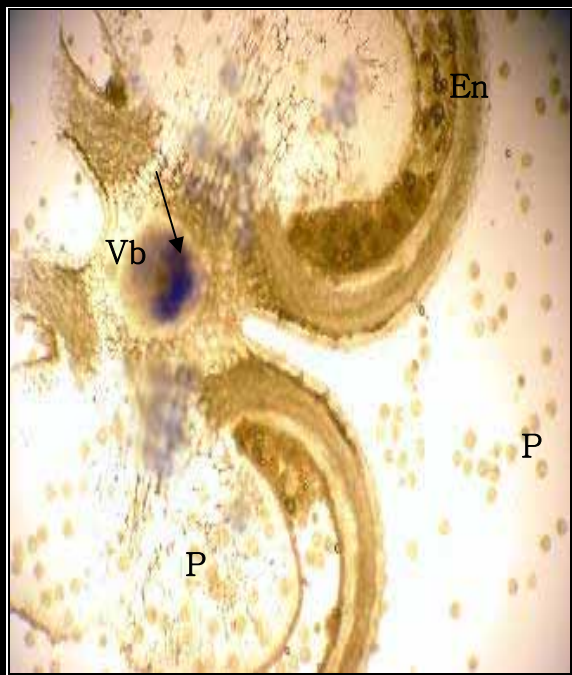
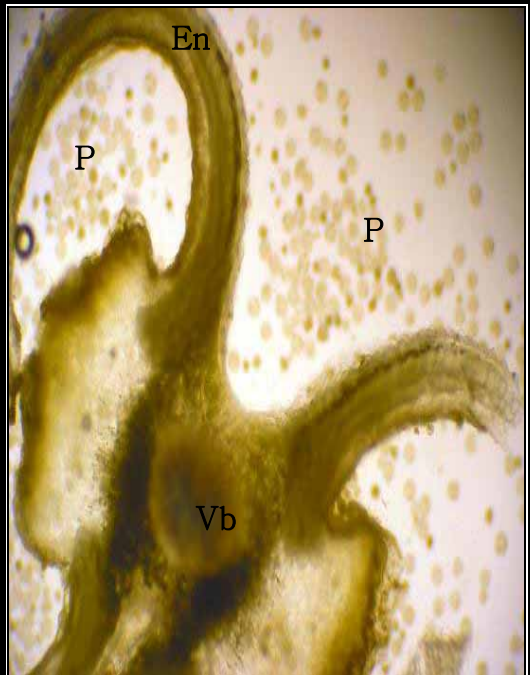
	
<p>Cross section of an anther in the first developmental stage (LM 10×10)</p>	<p>Cross section of an anther in the fifth developmental stage (LM 10×10)</p> <p>P : Pollen Vb : Vascular bundle En : Endothecium LM : Light Microscope</p>

Plate 5: Comparison of the reaction of anthers with NBT solution

The anther from stage one showed a strong blue colour on some of its tissues, especially the vascular bundle (Vb), while that of stage five hardly had any formazan deposits on its vascular bundle.


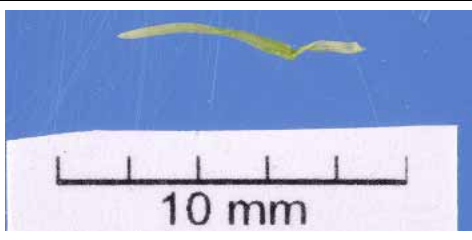

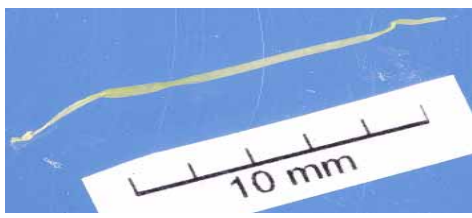

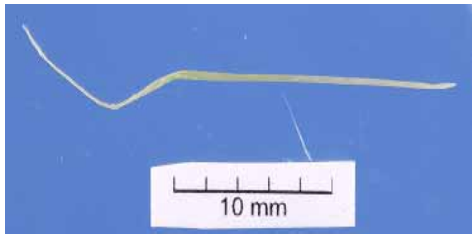
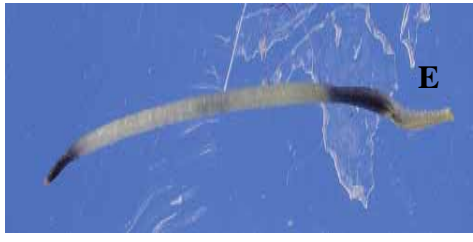
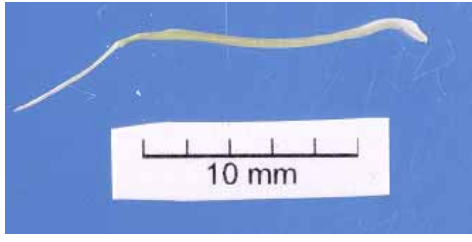
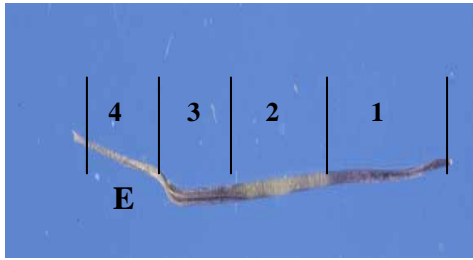
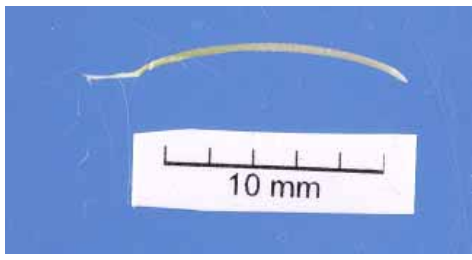
	The reaction with NBT solution	Control (no reaction with NBT)
STAGE 1	 A filament from Stage 1 showing a dark, curved shape. The part connected to the petal is labeled 'E'.	 A filament from Stage 1 showing a light, curved shape. A 10 mm scale bar is visible below it.
STAGE 2	 A filament from Stage 2 showing a dark, curved shape. The part connected to the petal is labeled 'E'.	 A filament from Stage 2 showing a light, curved shape. A 10 mm scale bar is visible below it.
STAGE 3	 A filament from Stage 3 showing a dark, curved shape. The part connected to the petal is labeled 'E'.	 A filament from Stage 3 showing a light, curved shape. A 10 mm scale bar is visible below it.
STAGE 4	 A filament from Stage 4 showing a dark, curved shape. The part connected to the petal is labeled 'E'.	 A filament from Stage 4 showing a light, curved shape. A 10 mm scale bar is visible below it.
STAGE 5	 A filament from Stage 5 showing a dark, curved shape. The part connected to the petal is labeled 'E'. The filament is divided into four segments labeled 1, 2, 3, and 4 from right to left.	 A filament from Stage 5 showing a light, curved shape. A 10 mm scale bar is visible below it.

Plate 6: Superoxide production in the filaments from five developmental stages of flower development (E : the part that was connected to the petal)

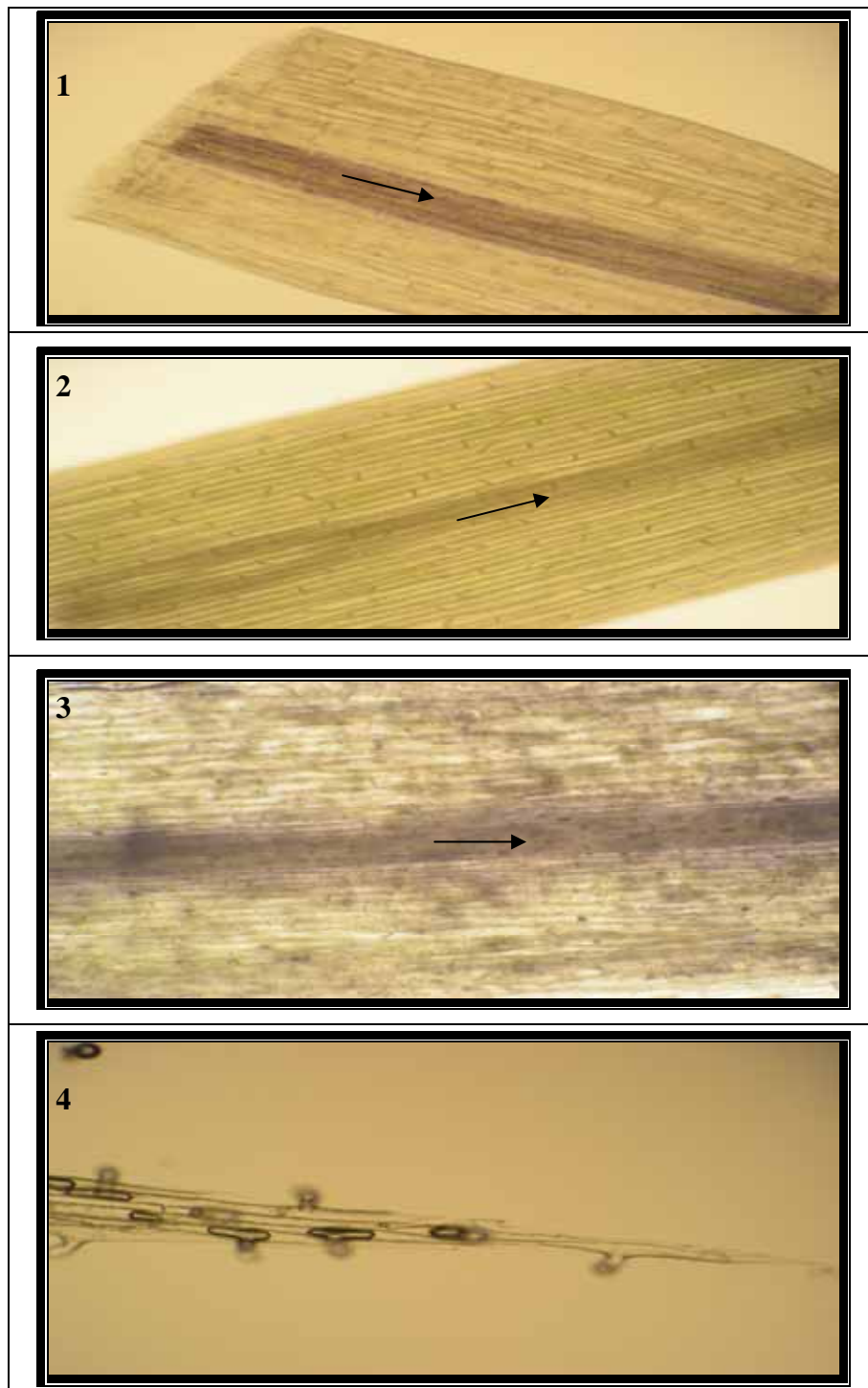


Plate 7: Longitudinal sections of petunia filaments (LM 10×10)

The number on the top left corner corresponds to the segment of filament from stage 5 shown in plate 6. The areas marked by arrows show blue formazan deposits particularly in the vascular bundles.

The fourth segment (the part connected to the petal) had no staining.

3.1.2 Superoxide production in pollen

After the pollen of the five developmental stages were soaked in a NBT solution and incubated for 24 hours, the total pollen number and stained pollen number were counted by using a haemocytometer under a light microscope (Plate 8). The total pollen number was the smallest at stage one, while stages four and five showed higher counts than at any other stage. The stained pollen number increased from stage one to stage four and five (Fig. 1).

The pollen grains harvested from the fifth stage were spread on germination medium, but there were no blue formazan deposits observable under a stereo microscope after 24 hours of incubation with a NBT solution.

Any changes in the superoxide production of pollen during a storage period was also investigated. Fresh pollen from the fifth stage displayed an intense blue colour from NBT reaction, whereas pollen after 10 days of storage in glass tubes hardly showed any blue colour at all.

Finally, the effect of heat over time on the superoxide production of pollen was observed. Pollen grains heated for 30, 60 and 90 seconds showed a yellow colour, while only the fresh pollen had an intense blue colour (Plate 9).

3.1.3 Control for NBT reactions

As a control for the NBT reactions resulting from superoxide, anthers, filaments and pollen grains were incubated in NBT solutions with or without 10 mM MnCl_2 . It is clear that only tissues reacted with only NBT in the absence of MnCl_2 resulted in a strong blue color (Plate 10).



Plate 8: Pollen grains with or without blue formazan deposits (LM, 10X10)

Stained pollen grains are marked with arrows.

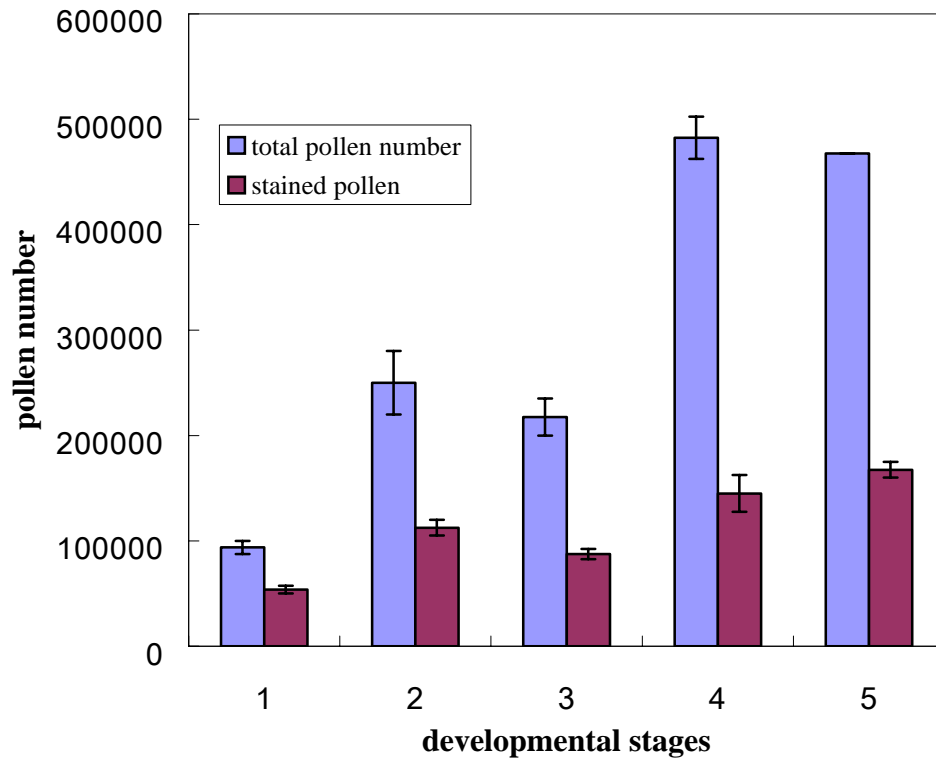


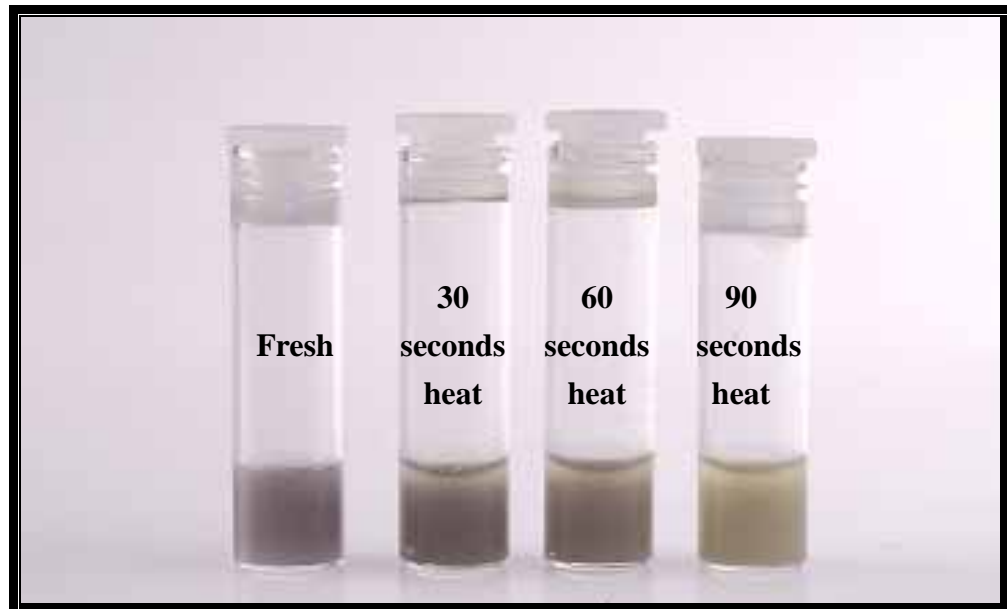
Figure 1: The number of pollen/flower and stained pollen number with blue formazan deposits at five developmental stages

The pollen grains were counted by using a haemocytometer under a light microscope (10×10). Error bars indicate standard error.

(A)



(B)



**Plate 9: (A)The change in superoxide production during storage of pollen
(B)The effect of heat on the superoxide production of pollen**







The reaction with NBT only solution	The reaction with MnCl ₂ /NBT solution
	
Anthers (stage 2)	
	
Filaments (stage2)	
	
Pollen (stage 5)	

Plate 10: Comparison between the results of the reaction with MnCl₂/NBT solution and the reaction with NBT only solution

3.2 Preliminary experiments for SOD assay

3.2.1 Storage temperature and period

The crude extracts from a stamen at the fifth stage of development were stored at both 4 °C and -20 °C. The SOD unit/flower at 4 °C decreased rapidly over a period ranging from two days to four weeks, while that at -20 °C remained stable (Fig. 2A).

The dehiscent anthers were also stored at both 4 °C and -20 °C for two days, one, two and four weeks before crude extracts were prepared for SOD activity determination. There were small decreases in the anthers weight over the storage period, but the changes in SOD activity were similar to that seen during the storage of the crude extracts at the two respective temperatures (Fig. 2B). As there was no significant difference in the total SOD activity when the crude extracts were stored at -20 °C for at least four weeks, this temperature was chosen to store crude extracts until required for further analyses.

3.2.2 Effect of pH on SOD activity and stability

Variation in pH level had a significant effect on SOD activity in the assay mixture. Enzyme activities at most pH levels were nearly 0, but pH 7.0 and pH 7.8 proved to be exceptions. The highest enzyme activities were at pH 7.8, and much less at pH 7.0, suggesting that pH 7.8 is optimal for SOD activity (Fig. 3A).

Stability of SOD activity in crude extracts held at different pH levels before SOD determination was also tested. Increasing SOD activity was found when pH of the crude extracts was changed from 3.0 to pH 7.8 but lower SOD activity resulted when the pH of the crude extracts was 8.5 or higher (Fig. 3B).

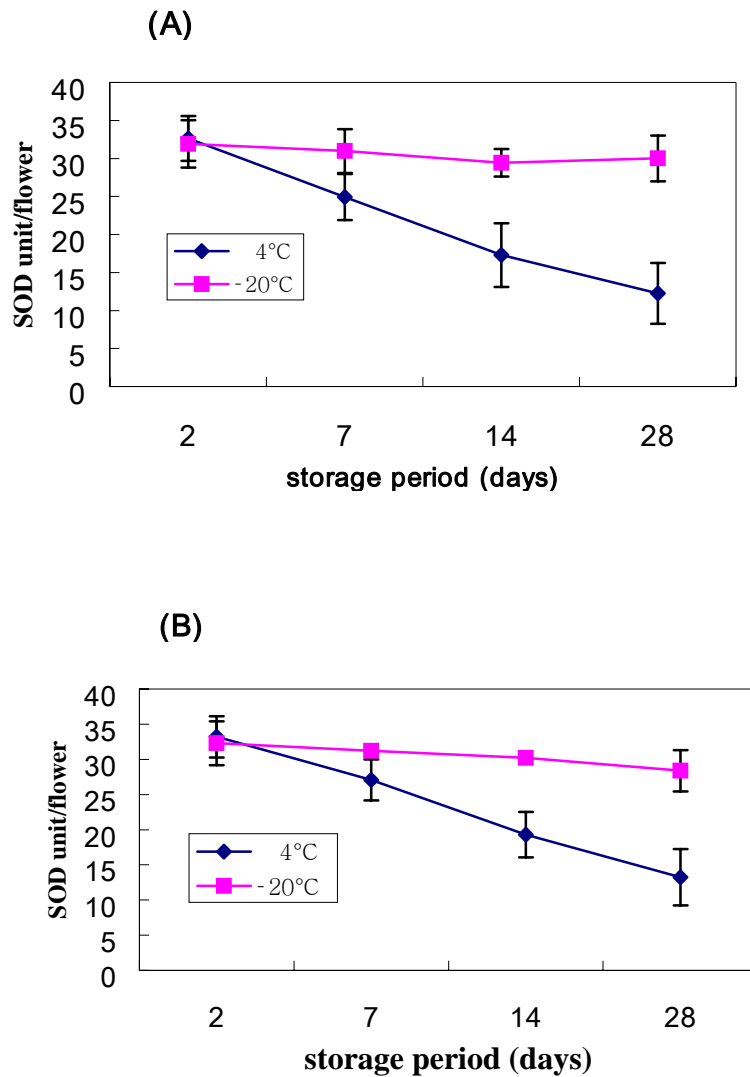


Figure 2: SOD activity at different storage periods and temperatures

(A) The mean SOD unit/flower of crude extract

(B) The mean SOD unit/flower of dehiscent anthers at different storage periods and temperatures Error bars show standard error of the mean values of three different groups of flowers.

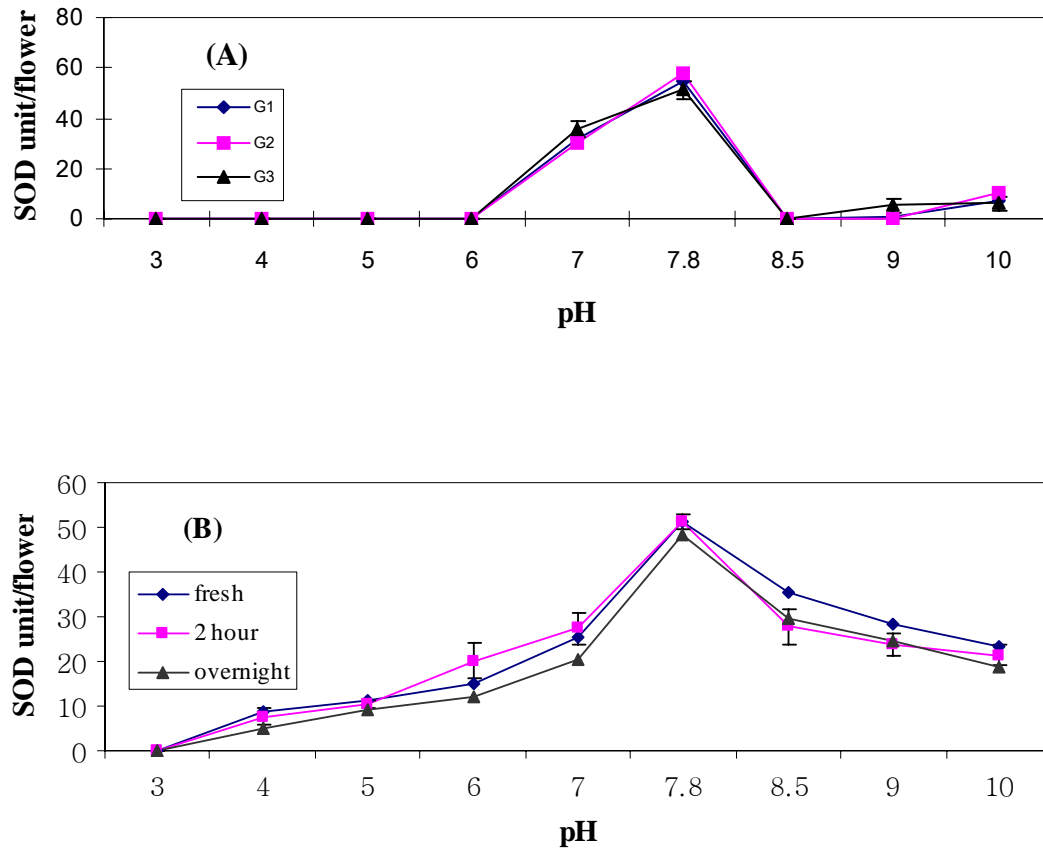


Figure 3: (A) Effect of pH of the enzyme assay mixture on SOD activity

The graph shows the change of SOD activity at different pH levels for three different groups. G1~G3 represent groups of flowers collected from different pots on different days. Error bars indicate standard error.

(B) Effect of pH of the crude extracts on the stability of SOD activity

The change of SOD activity at different pH levels in three different groups (freshly prepared extracts, 2 hours and overnight) of assay mixture was investigated with three replicates over different periods. Error bars show the standard error.

3.3 SOD activity in the male reproductive parts of petunia

3.3.1 Developmental changes in SOD activity

The petunia flowers were divided into five developmental stages of growth and maturity in the stamen and SOD activity was separately investigated in the different parts of these stamens. The amount of SOD unit per flower and total activity was calculated from enzyme assay data and one unit of SOD was defined as the level of enzyme activity that inhibits NBT photoreduction by 50%. The experiment was repeated three times, with three replicates at each stage tested in each experiment.

3.3.1.1 SOD activity in the extracts of the anther

SOD activity in the crude extracts of the anther (Fig. 4) varied significantly among the 5 stages (ANOVA: $F_{2,5}=127.74<202$, $P=0.00<0.05$). SOD activity in the crude extracts of petunia anthers at all five stages was much higher than that of the filaments (see Fig. 4 and Fig. 5). In the crude extracts of the anther, the highest SOD unit/flower was at stage two (67.993), followed by stage one, three and four, while the least SOD unit/flower activity was at stage five (32.805). There are three groups from the T-test (Tukey HSD All-Pairwise Comparisons Test) in which the means are not significantly different from one another.

3.3.1.2 SOD activity in the extracts of the filament

SOD activity in the crude extracts of the filament (Fig.5) varied significantly among the 5 stages (ANOVA: $F_{2,5}=101.24<202$, $P=0.0364<0.05$). SOD unit/flower in the filaments at the five developmental stages was very low. The SOD unit/flower from stage one to stage four remained at 10 and at stage five it declined to 7.637. From the T-test, values with the same letter represent statistically homogenous groups.

3.3.1.3 SOD activity in the extracts of the stamen

SOD activity in the crude extracts of stamen varied (Fig. 6) significantly among the 5

stages (ANOVA : $F_{2,5}=102.87<202$, $P=0.00<0.05$). The SOD unit /flower of stamens was similar to the sum of the SOD activity in the anthers and filaments. Therefore, the developmental change of SOD unit /flower in stamen was similar to that of the anthers. The highest SOD unit /flower was found at stage two (72.400), followed by stage one (64.990), stage three (59.022) and stage four (44.047), while the lowest SOD unit/flower was in stage five (35.644). From the T-test, values with the same letter represent statistically homogenous groups.

3.3.1.4 SOD activity in the extracts of the pollen

SOD activity in the crude extracts of the pollen varied (Fig. 7) significantly among the 5 stages (ANOVA: $F_{2,5}=22.277<202$, $P=0.0257<0.05$). The SOD unit /flower of the pollen grains was very low (see Fig. 7 and Fig. 8). Nevertheless, SOD activity in the pollen increased from stage one to stage four (4.784), while it decreased at stage five (2.102). From the T-test, values with the same letter represent statistically homogenous groups.

3.3.1.5 SOD activity in the extracts of the anther minus pollen

SOD activity in the crude extracts of the anther minus pollen (Fig. 8) varied significantly among the five stages (ANOVA: $F_{2,5}=28.910<202$, $P=0.00<0.05$). The SOD unit /flower of anthers without pollen grains was very high. The highest SOD unit /flower was at stage two (63.754), followed by stage one (57.272), stage three (50.137) and stage four (33.140). while the least SOD unit/flower activity was at stage five (25.210). The letters from the T-Test, values with the same letter represent statistically homogenous groups.

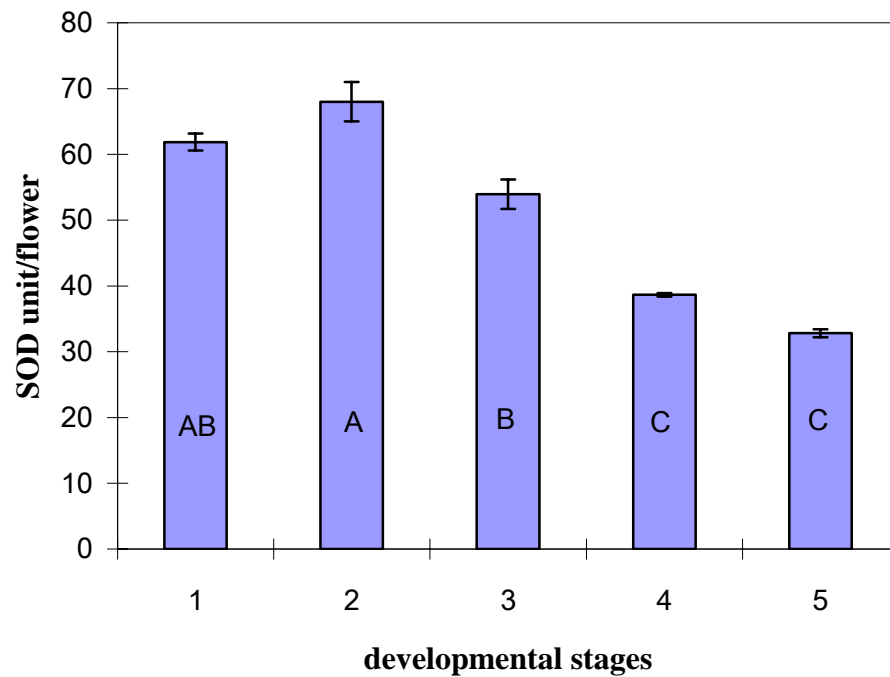


Figure 4: The mean SOD unit/flower of anthers of the petunia at all five stages Data are from three groups with the mean value taken of the three replicates. The experiment was repeated three times with similar results. Error bars indicate the standard error of the mean. The letters represent analysis of the results using T-test (Tukey HSD All-Pairwise Comparisons Test).

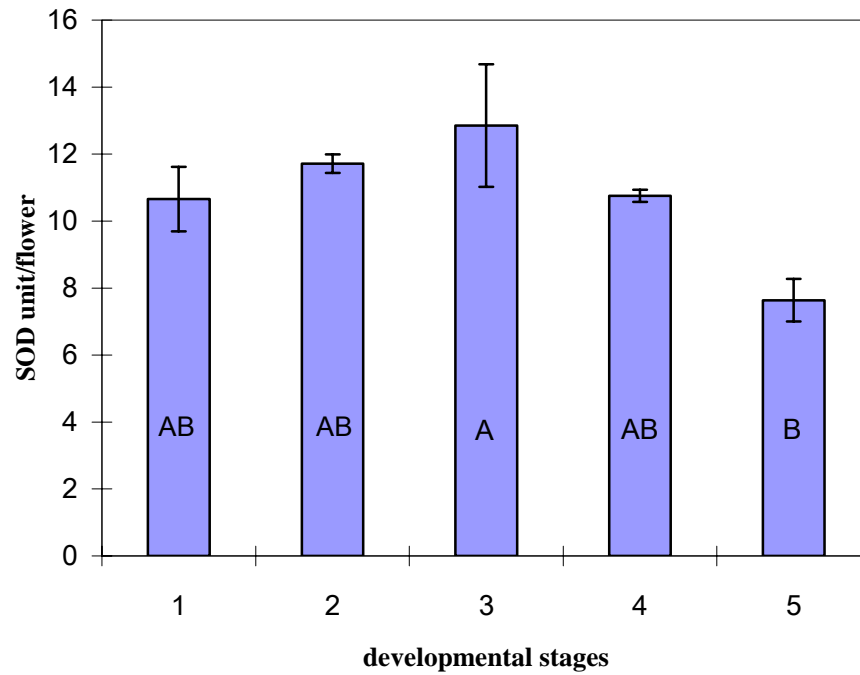


Figure 5: The mean SOD unit/flower in the filaments of five developmental stages Data are from three groups with the mean value taken of the three replicates. The experiment was repeated three times with similar results. Error bars indicate the standard error of the mean. The letters represent analysis of the results using T-test (Tukey HSD All-Pairwise Comparisons Test).

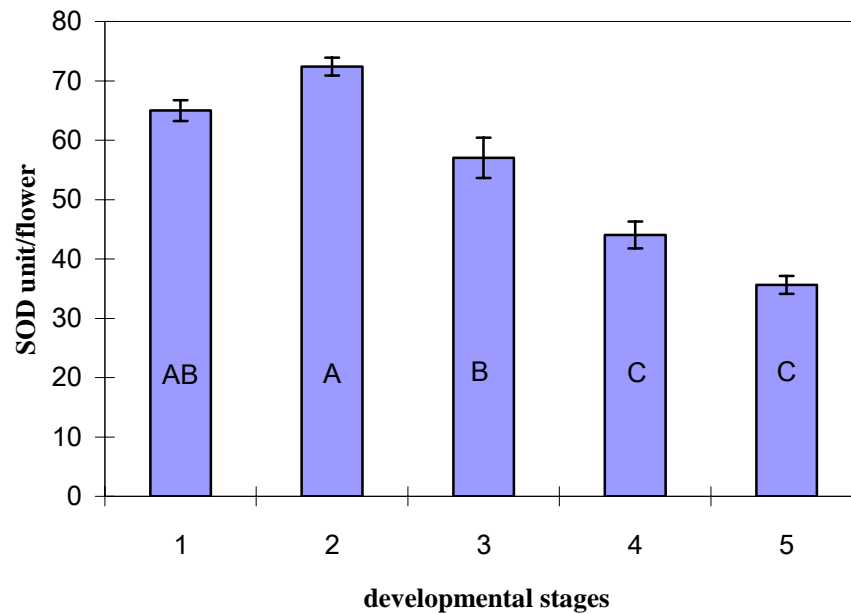


Figure 6: SOD unit/flower in the stamens of five stages

Data are from three groups with the mean value taken of the three replicates.

The experiment was repeated three times with similar results. Error bars indicate the standard error of the mean. The letters represent analysis of the results using T-test (Tukey HSD All-Pairwise Comparisons Test).

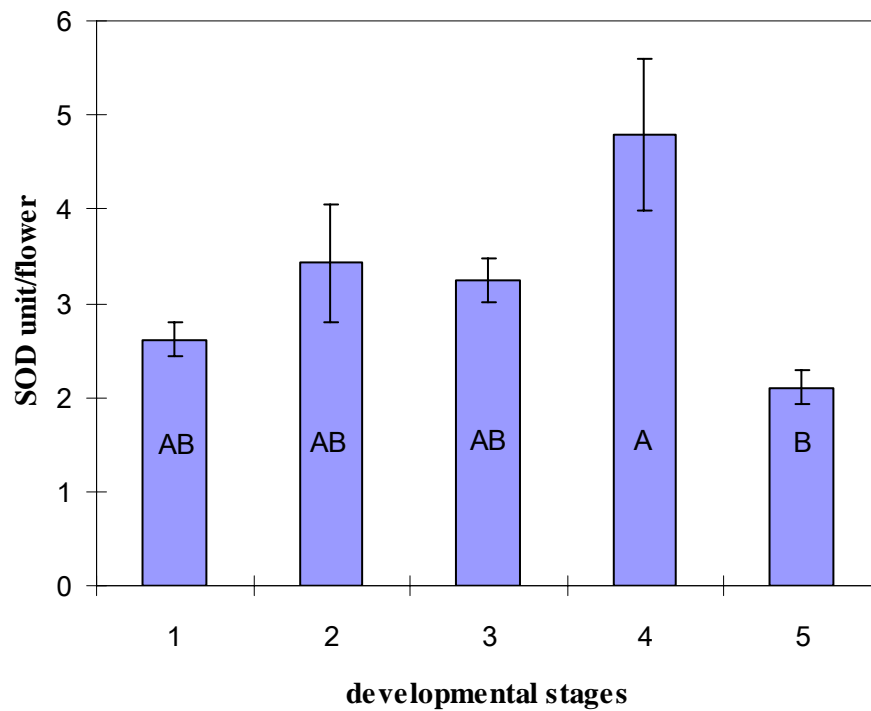


Figure 7: The mean SOD unit/flower in the pollen grains of five developmental stages Data are from three groups with the mean value taken of the three replicates. The experiment was repeated three times with similar results. Error bars indicate the standard error of the mean. The letters represent analysis of the results using T-test (Tukey HSD All-Pairwise Comparisons Test).

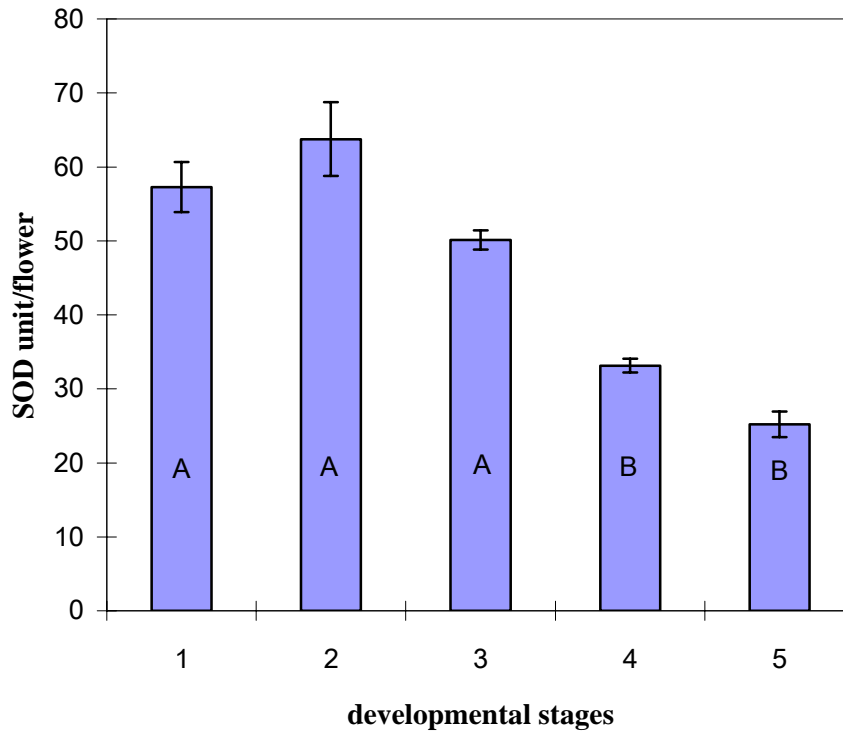


Figure 8: The mean SOD unit/flower of anthers minus pollen at all five stages Data are from three groups with the mean value taken of the three replicates. The experiment was repeated three times with similar results. Error bars indicate the standard error of the mean. The letters represent analysis of the results using T-test (Tukey HSD All-Pairwise Comparisons Test).

3.4 Isozyme analysis of SOD within stamens at five different stages of development (native PAGE)

Polyacrylamide gel electrophoresis (native PAGE) was performed and superoxide dismutase was localized. Three distinct forms of the enzyme in crude extracts of stamens, anthers, filaments and pollen grains (from 20 flowers, respectively) were detected. The third fastest migrating band appeared to be of the least active of these SOD isozymes in the crude extracts of the different tissues. These bands were expressed in order of increasing relative mobility (Plate 11). Then on one gel, the same bands from different tissues, with their apparent quantitative differences depending on the particular stage of development were exhibited. This analysis showed that the bands from stages two to four are stronger than those from stages one and five. In this process a fourth band (★) also appeared, but the fact that this remained after boiling and disappeared following dialysis, suggested that it may not have been an SOD enzyme (Plate 11).

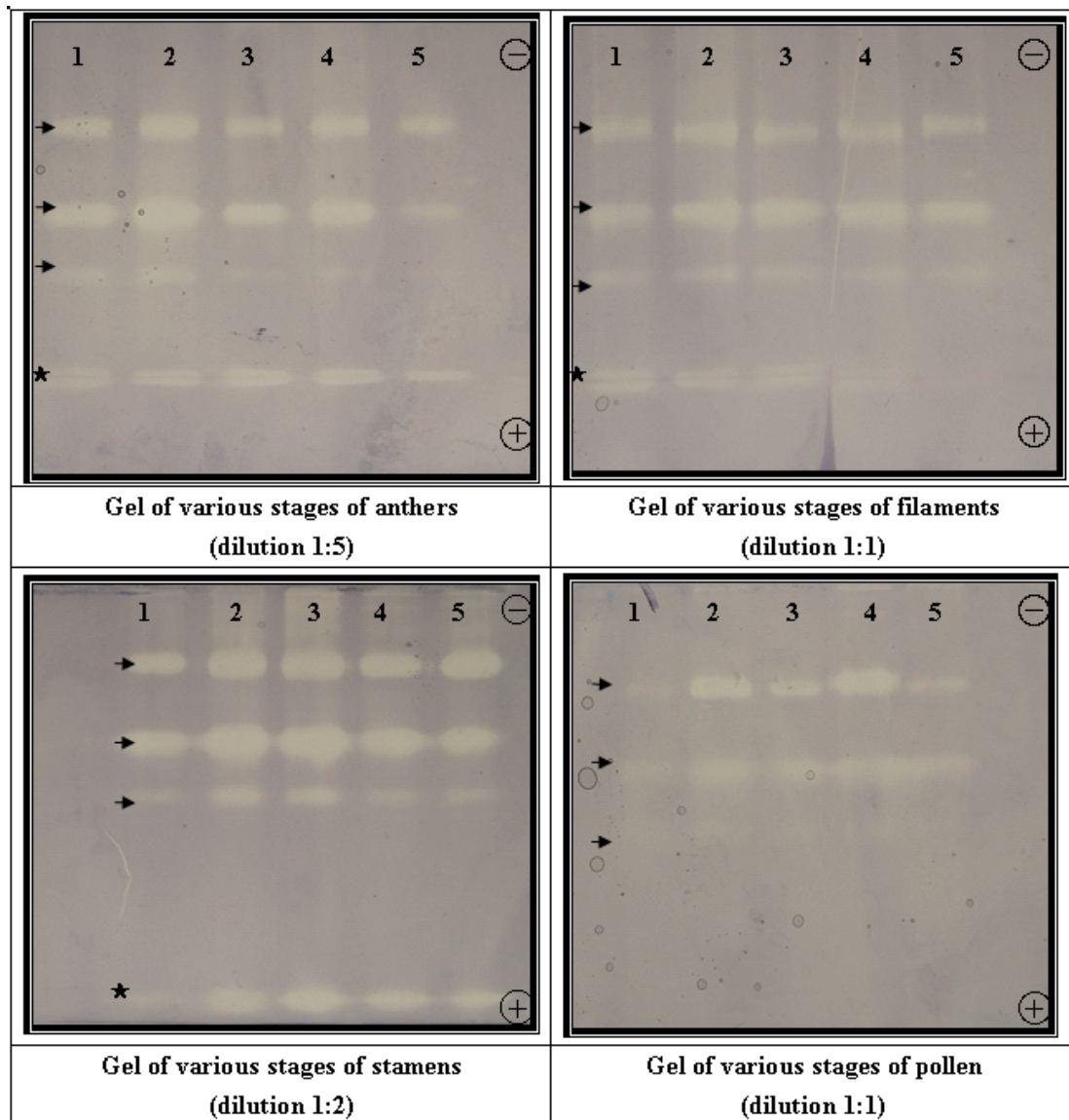


Plate 11: Non-denaturing polyacrylamide gel electrophoresis of crude extracts from petunia flowers of five developmental stages

All the lanes were loaded with 10 μ l (enzyme extract : 60 % sucrose =2:1) and the enzyme extract was diluted with the extraction buffer. From the left, lane 1: developmental stage one, lane 2: stage two, lane 3 : stage three, lane 4 : stage four, lane 5 : stage five.

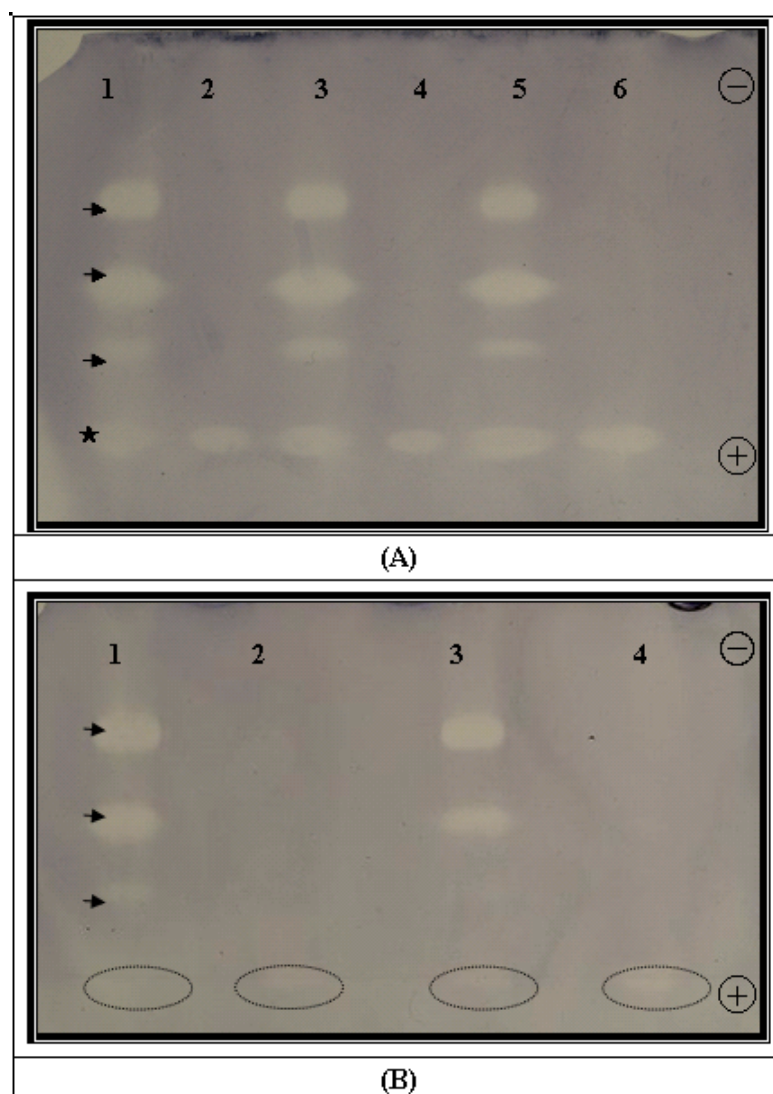


Plate 12: (A) Comparison of the crude extracts before and after boiling of the stamen from the fifth developmental stage of petunia flowers

From the left, lanes 1, 3 and 5 : before boiling of crude extracts,
lanes 2, 4 and 6 : after boiling.

(B) Comparison of the crude extracts after boiling and dialysis of the stamen from the fifth stage stamen

From the left, lanes 1 and 3 : crude extracts after dialysis,
lanes 2 and 4 : boiling and dialysis.

After dialysis, the fourth band almost disappeared in all the lanes.
Dotted circles highlight the missing bands.

3.5 Isolation and purification of SOD

using ion exchange chromatography

3.5.1 Optimization of protein precipitation

Firstly, there was an addition of ammonium sulphate to remove unwanted proteins in a crude extract from 300 stamens (60 flowers of the fifth stage) in three steps. The pellet produced after centrifuging at each step, was dissolved in a 10 ml extraction buffer. Following this the SOD activity and protein content of the supernatant and pellet after dialysis at each step was determined. It was found that the supernatant with a 30 - 60% addition showed a higher purification than from any other steps. Unfortunately, however, the protein content was too low for ion exchange chromatography.

Table 2: Results after the addition of ammonium sulphate in 3 steps

		Total activity (units)	Total protein (mg protein)	Specific activity (unit / mg protein)	Purification (fold)	Recovery (%)
crude extract	supernatant	216.0	20.7	10.4	1	100
0 - 30%	supernatant	21.6	3.3	6.4	0.6	10.0
	pellet	126.7	12.7	9.9	0.9	58.6
30 - 60%	supernatant	25.6	0.5	48.7	4.6	11.8
	pellet	92.7	8.3	11.1	1.0	42.9
60 - 90%	supernatant	29.1	0.6	46.4	4.4	13.4
	pellet	78.5	15.7	4.9	0.4	36.3

Secondly, there was a direct addition of a solution saturated with 30%, 40%, 50%, 60%, 70%, 80% and 90% ammonium sulphate into the crude extract of 300 stamens (60 flowers of 5th stage). After centrifuging and dialysis, SOD assay and protein determination were conducted (Fig. 9). The additions of over 50% saturation showed high enzyme specific activity, but low protein content, and so 40% saturation with ammonium sulfate was chosen for ion exchange chromatography to measure SOD activity and protein content.

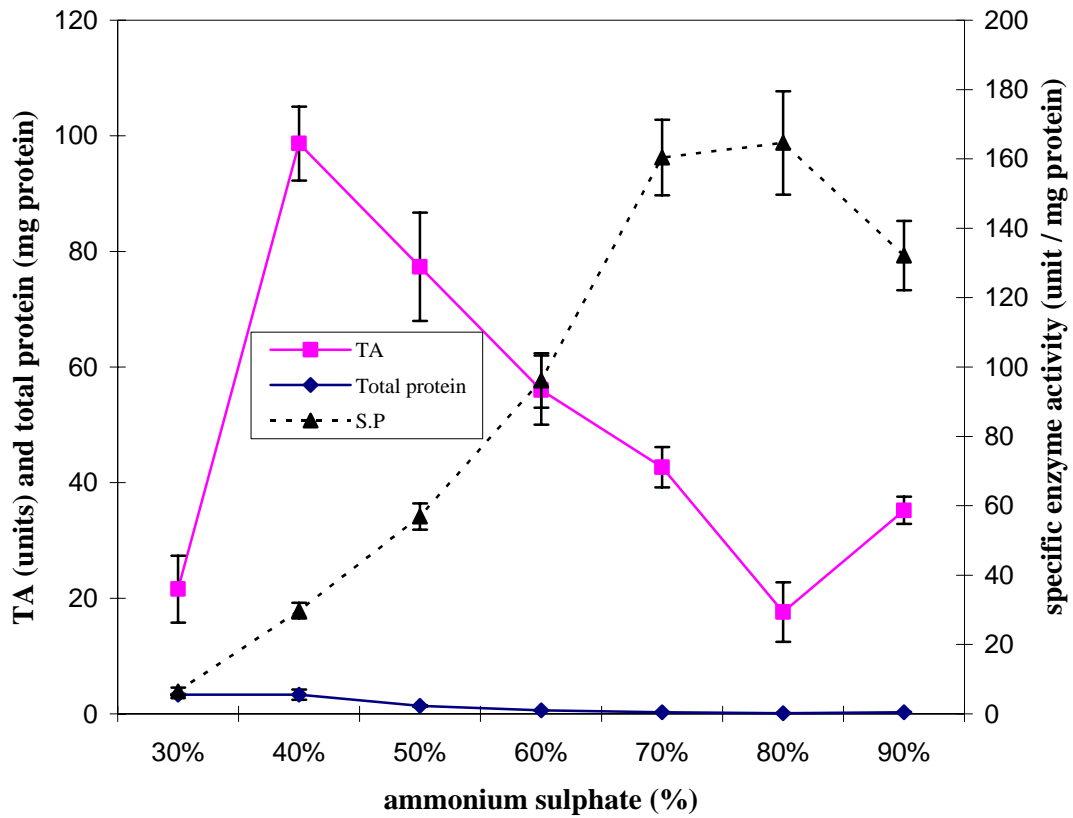


Figure 9: Variation in total activity (TA), total protein and specific enzyme activity (S.P) with different ammonium sulphate percentages.

The experiment was repeated three times with similar results.

Error bars indicate standard error of the mean.

3.5.2 Purification of SOD enzyme

After the ammonium sulphate precipitation processes, further purification of SOD was achieved through ion exchange chromatography. A dialyzed and concentrated sample was applied to a 1.5 cm × 25 cm column of DEAE cellulose, washed with buffer (0.01 M potassium phosphate buffer, pH 7.8) and 40 fractions collected. Next, a linear gradient (0.05 M NaCl - 0.5 M NaCl) in the same buffer was applied to the column and another 40 fractions were collected. SOD was found in the fractions numbered 11 - 17 and 59 - 66, revealing two peaks (Fig. 10). The fractions with higher peak SOD activity showed both high specific enzyme activity and high levels of purification (Table 3). The specific activity of purified SOD in the bound fraction was 66.5 U/mg and the yield of the total activity was 3.0%. When examined by native PAGE, the bound protein fraction showed only one SOD isozyme band, while crude extract, the supernatant with ammonium sulphate (0 - 40%) and the unbound protein fraction commonly presented three bands on a native PAGE Gel (Plate 13). The progress of enzyme purification was also monitored by SDS-PAGE. There were six major bands in both the crude extract and the supernatant from the ammonium sulphate addition step, while the unbound fraction from the DEAE cellulose column had four bands and the bound fraction showed two (Plate 14).

3.5.3 Characterization of the SOD in the bound fraction

3.5.3.1 The effect of pH

SOD activity in the bound fraction was optimal in the buffer at pH 7.8 and the activity was retained after 24 hours incubation in the buffers at pH from 6 to 9 (Fig. 11).

3.5.3.2 The effect of temperature and storage period

The SOD activity of the purified enzyme in the bound fraction was stable at -20 °C over a period of four weeks, while the activity at 4 °C decreased rapidly (Fig. 12).

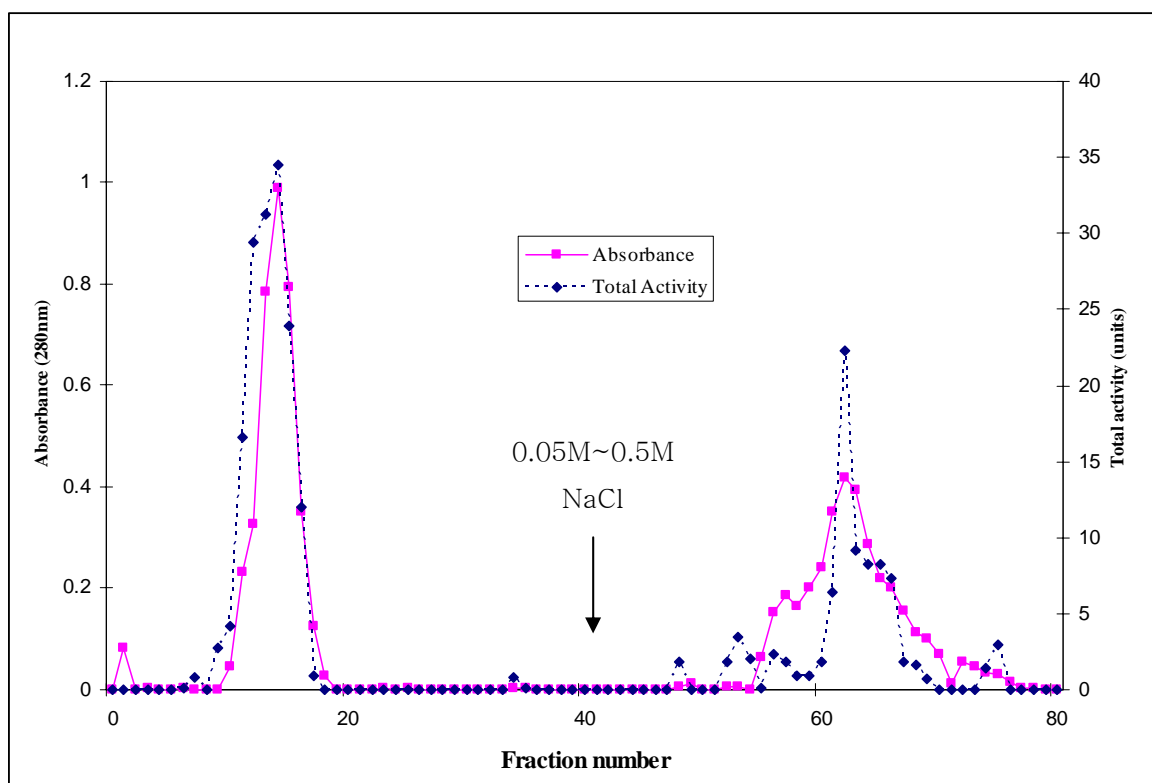


Figure 10: Total SOD activity and protein content (absorbance at 280 nm) of the fractions from DEAE cellulose anion exchange chromatography

The column was equilibrated with 0.01 M potassium phosphate buffer (pH 7.8). The first 40 fractions were eluted with 0.01 M potassium phosphate buffer (pH 7.8) and the next fractions (41 - 80) were eluted with a linear 0.05 M - 0.5 M NaCl gradient shown as a dotted line. Each fraction of 2.2 ml were measured for absorbance (280 nm) and assayed for SOD activity.

Table 3: Purification of superoxide dismutase from 1,000 petunia stamen

Purification step	Total activity (units)	Total protein (mg protein)	Specific activity (unit / mg protein)	Purity factor (fold)	Recovery (%)
Crude extract	725.0	40.4	17.9	1.0	100
Ammonium sulphate fraction (0 – 40%)	225.1	9.6	23.4	1.3	31.0
DEAE cellulose column unbound fraction (#14)	34.5	0.6	57.5	3.2	4.7
DEAE cellulose column bound fraction (#62)	22.2	0.3	66.5	3.7	3.0

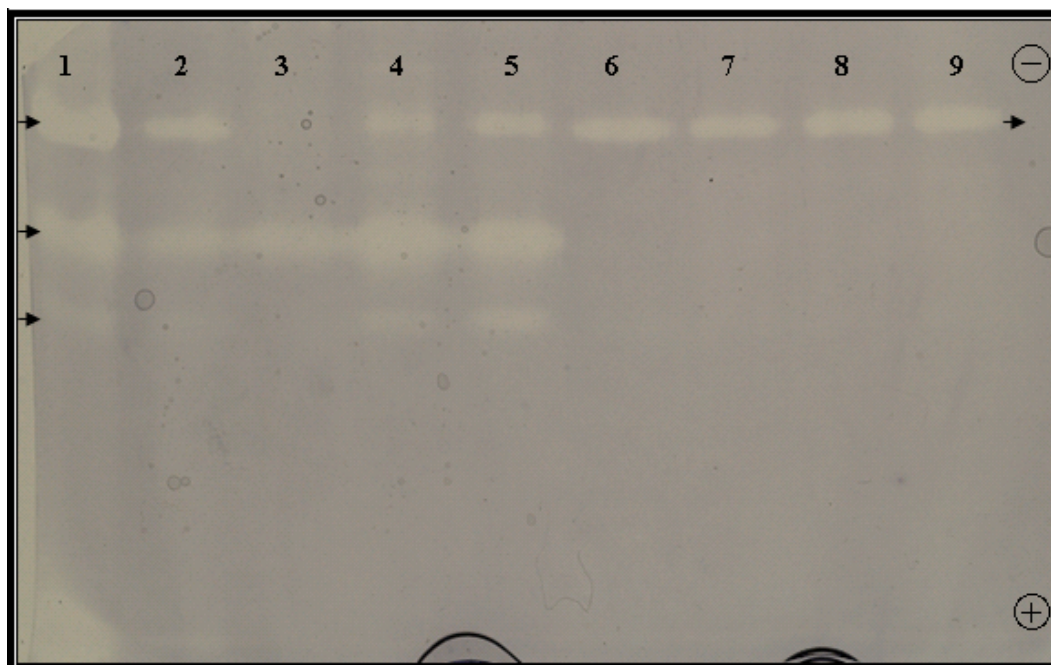


Plate 13: Detection of SOD isozymes after native gel electrophoresis of petunia stamen extracts at various stages of enzyme purification

All the lanes showed three bands except for the bound fractions lanes from number 6 to number 9, which had only one band.

From the left, lane 1 : crude extract of 1,000 stamen (dilution 1:8),

lane 2 : after adding ammonium sulphate(0 - 40%) and dialysis (dilution 1:6)

lanes 3, 4 and 5: unbound fractions (No. 13, 14 and 15) with high SOD activity after ion exchange chromatography on DEAE cellulose (no dilution)

lanes 6, 7, 8 and 9 : bound fractions (No. 61, 62, 63 and 64 with high SOD activity after ion exchange chromatography on DEAE cellulose (no dilution).

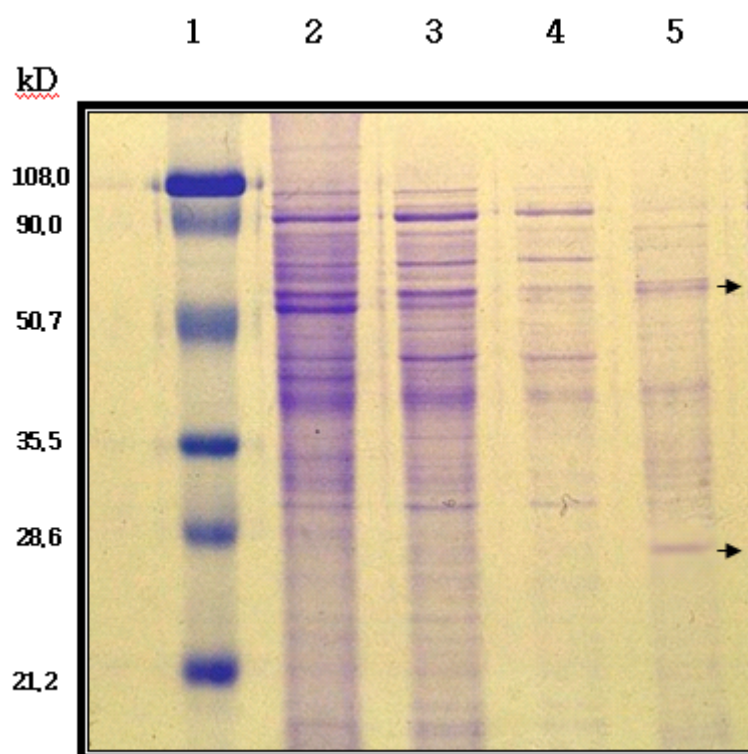


Plate 14: Protein samples at various steps of enzyme purification on a 12% SDS-PAGE gel

From the left, lane1: BioRad pre-stained low-molecular weight protein markers were used (5 $\mu\ell$)

lane2 : crude extract from 1,000 stamen

lane 3 : after the addition of ammonium sulphate 0 - 40%

lane 4 : unbound fraction (#14) after ion exchange chromatography

lane 5 : bound fraction (#62) after ion exchange chromatography.

Loaded samples, except lane 1, were mixed with SDS sample buffer as a ratio of 4:1, then boiled for 5 minutes. Ten $\mu\ell$ were loaded in each lane (except for lane1).

The mg protein content of all samples was similar to that of the bound fraction (0.328 mg).

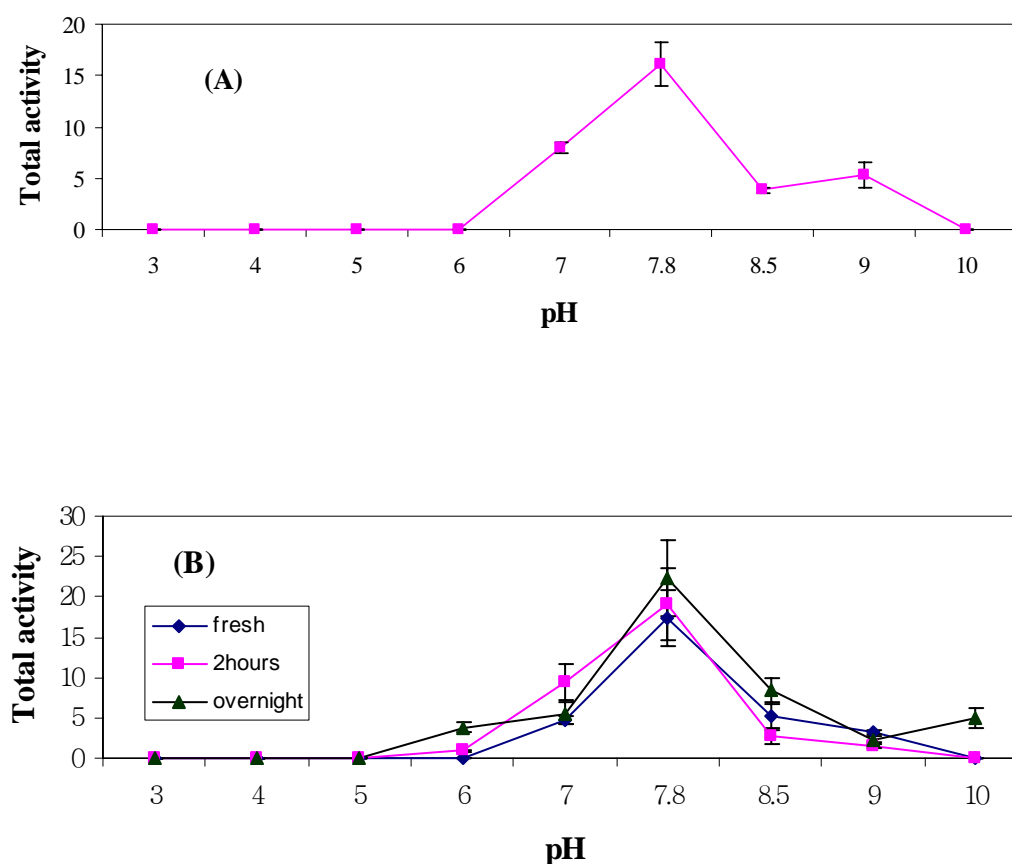


Figure 11: The effect of pH on the purified enzyme

(A) The effect of varying the pH of the enzyme assay mixture

(B) The effect of pH of the enzyme solution before SOD assay on its stability

The DEAE cellulose column chromatography step was repeated three times, each started with crude extracts of 1,000 stamens as described in 2.6.2. The bound fraction with the highest SOD activity in each replicate experiment was used for this experiment on the effect of pH on SOD activity. Each data point shown is the mean \pm SE of the SOD activity in the three fractions used.

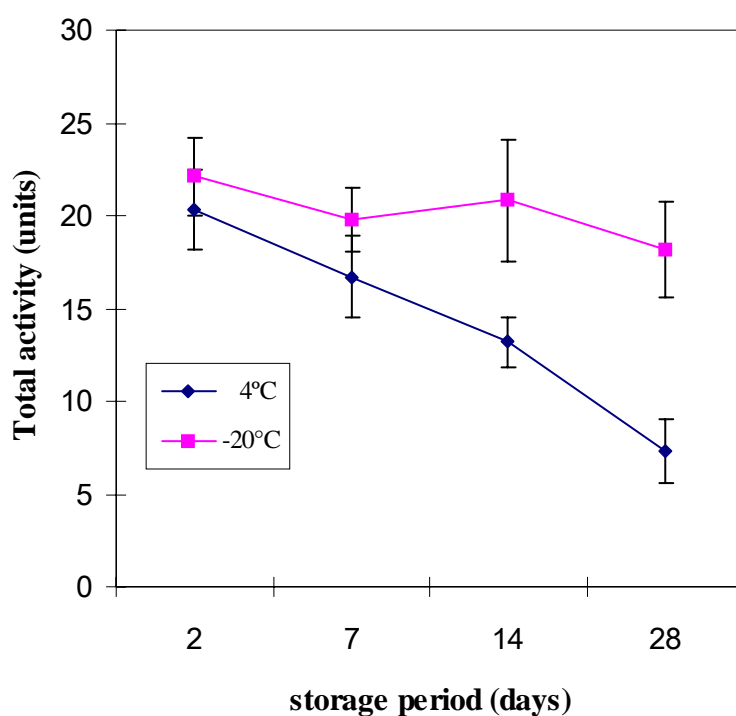


Figure 12: The effect of temperature and storage period on the SOD activity in the bound fractions from the DEAE cellulose column

The DEAE cellulose column chromatography step was repeated three times, each started with crude extracts of 1,000 stamens as described in 2.6.2. The bound fraction with the highest SOD activity in each replicate experiment was used for this experiment on the effect of temperature and storage period on SOD activity. Each data point shown is the mean \pm SE of the SOD activity in the three fractions used.

3.5.3.3 Effect of inhibitors

The effect of two inhibitors, cyanide and hydrogen peroxide, on both crude extract and purified enzymes was examined. In normal native gel (Plate 15 C), the bound fraction of the purified enzyme showed only the first band, while the others, including the unbound fraction, commonly showed three bands. However, the first band of the unbound fraction was very faint.

After cyanide treatment (Plate 15 A), the crude extract, the supernatant with addition of 0 - 40 % ammonium sulfate and unbound fraction commonly displayed two bands (the first and second band) whereas the bound fraction still retained only one band (the first band).

With the hydrogen peroxide treatment, the crude extract, the supernatant, the unbound and bound fractions all had only exhibited the first band (Plate 15 B).

The effects of these inhibitors seemed to show that the purified enzyme was an Mn SOD. The first band of the crude extract was an Mn SOD, which was not sensitive to either cyanide and hydrogen peroxide. The second band which was sensitive to H_2O_2 , but not sensitive to cyanide was a Fe SOD. The third band showed sensitivity to both cyanide and H_2O_2 , suggesting that it was a Cu/Zn SOD.

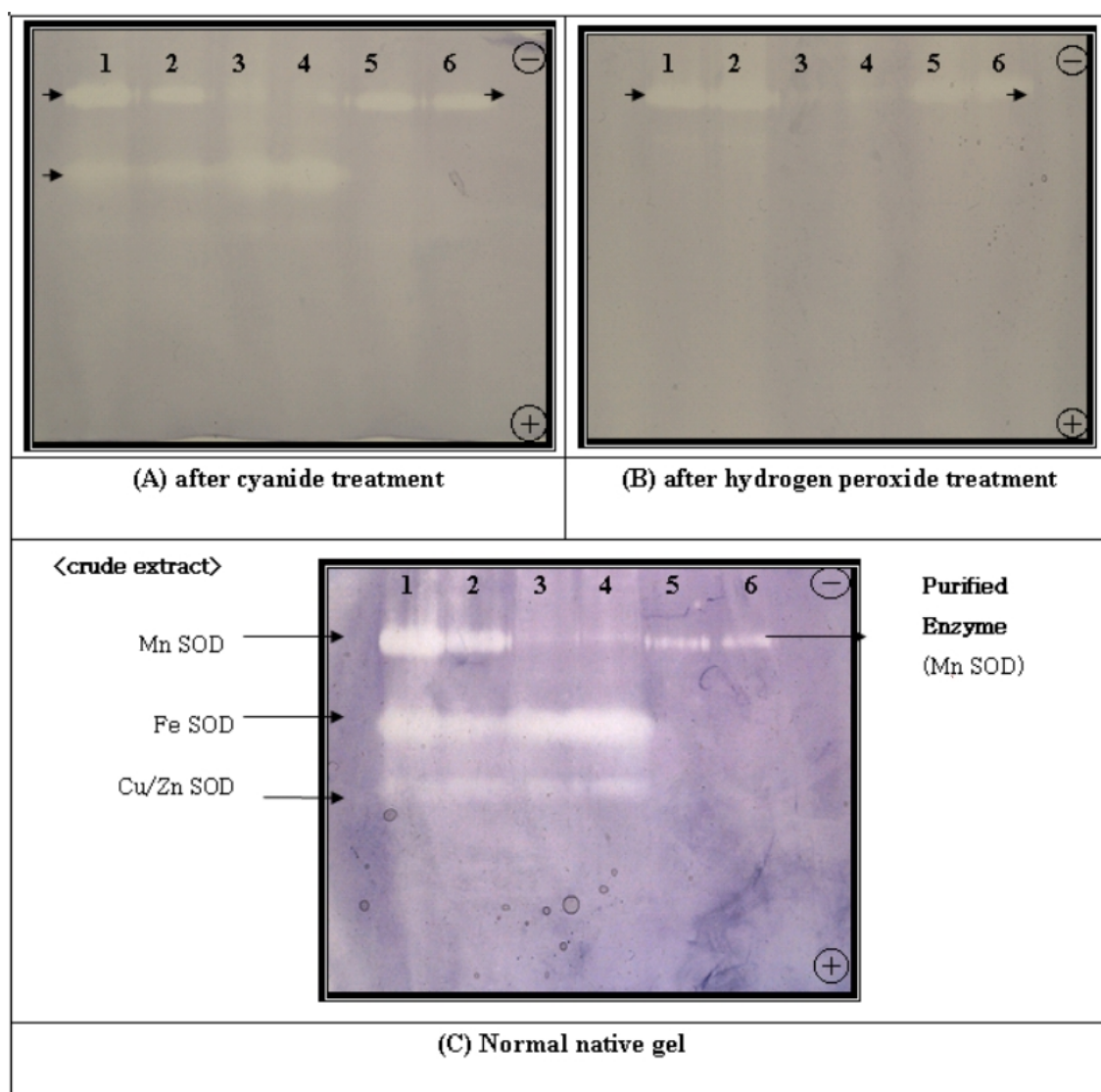


Plate 15: Comparison of SOD isozymes on native polyacrylamide gels treated with or without H_2O_2 (5 mM) or KCN (3 mM)

From the left, lane1: crude extract, lane2: the supernatant with 0 - 40% ammonium sulphate, lanes 3 and 4: unbound fraction No.14 and No.15, lanes 5 and 6: bound fraction No.62 and No.63.

CHAPTER 4

DISCUSSION

4.1 Overview and significance of findings

Initially, both superoxide production and the occurrence of SOD in the male reproductive parts of the petunia were studied. The results showed that when the anthers, filaments and pollen from five developmental stages were soaked in NBT solution for 24 hours, superoxide production at stage one (flower bud) or two (corolla extending) was usually high in both anthers and filaments, while there was weak or no superoxide production at stage five (petals fully open and anthers dehiscent). Similarly, SOD activities were also at their highest in the anthers and filaments of stage one or two, and at their lowest in those of stage five. However, with regard to pollen over the five developmental stages, superoxide production and SOD activity were at their highest at stage four (petals fully open but anthers predehiscent), suggesting that the metabolic activity of pollen is also high at this stage. Moreover, anthers, filaments and pollen all commonly show a positive correlation between their superoxide production and their SOD activity. It was also noticeable that most SOD activities in the stamen were localized in anthers including the pollen, suggesting that the filaments have more of a support, rather than metabolic, function.

SOD was also examined in terms of the isozymes present within the stamen of five developmental stages. In this phase of the experiment, three isozymes were commonly found in the anthers, filaments and pollen. More specifically, when inhibitors were used, these parts of the plant exhibited the Mn SOD, Fe SOD and Cu/Zn SOD isozymes. This is the first time three different SOD isozymes have been reported in an extract from the male reproductive parts of a flower. Particularly surprising is the appearance of Fe SOD, as earlier studies have argued that it is not common in higher plants (Alche *et al.* 1998). Furthermore, it is also noticeable that the Mn SOD and Fe SOD bands on the native PAGE

gels are stronger, suggesting that the activities of Mn SOD and Fe SOD are higher, than those in the Cu/Zn SOD bands. Complete characterization of the different isozymes will have to await future research.

For the next phase of the research, SOD from 1,000 stamens of dehiscent mature flowers was partially purified through ammonium sulphate fractionation and ion exchange chromatography. After purification, the bound protein fraction showed only one band after a native PAGE, which appears to be a Mn SOD, as it is sensitive to neither hydrogen peroxide nor cyanide. Therefore, the purification protocol used had enabled the separation of this SOD isozyme from the other isozymes. The specific activity of the purified SOD in the bound fraction was 66.5 U/mg and the yield of total activity was 3.0%. The progress of enzyme purification was monitored using SDS-PAGE and the bound fraction contained two major bands, suggesting that further purification steps are required to achieve purification of the Mn SOD to homogeneity. The characteristics of the partially purified SOD in the bound fraction remained unchanged compared with those of the crude extracts.

4.2 Superoxide production and SOD activity in petunia stamens

An NBT-based method is useful for detecting superoxide production, because the small NBT molecule can easily diffuse into the cell wall. Furthermore, the insolubility of its reduced formazan is very helpful in localizing superoxide radicals, even though it does not allow for the measurement of the yield of those radicals. Despite these strengths, however, other methods of analysis could have been used, including electron spin resonance, chemiluminescence and the use of the redox protein Cyt *c* to detect and monitor superoxide radicals. These methods, however, all have potential drawbacks. Electron spin resonance needs expensive infrastructure and has problems with signal specificity. Chemiluminescence cannot directly measure superoxide radicals, and Cyt *c* reduction assays have not been suitable for plant cells because of problems with diffusion in the cell wall (Able *et al.* 1998).

When anthers, filaments and pollen grains were incubated in NBT solutions, both with and without 10mM MnCl₂, for the purpose of establishing a control for the NBT reactions

resulting from superoxide, only the tissues presented a strong blue colour in the absence of MnCl_2 (Plate 10). These results confirmed that the area and amount of blue formazan deposits on the tissues were good indicators of the exact location and the quantity of superoxide production.

The production of superoxide has been reported in neutrophils, monocytes and macrophages (Halliwell and Gutteridge 1985), chloroplasts (Asada 1984), mitochondria (Boveris *et al.* 1984), microsomes (Kuthan and Ullrich 1982) and nuclei (Patton *et al.* 1980). Moreover, a recent study has reported that superoxide production is mainly controlled by wall-bound peroxidases and membrane-bound NADP oxidases, especially with regard to the developmental programme (Jones and Smirnov 2005). The variation of superoxide production at the five developmental stages of the petunia stamen could, therefore, be considered as a result of the activities of both wall-bound peroxidases and membrane-bound NADP oxidases against growth and developmental oxidative stresses. In anthers and filaments (Plates 4 and 6), the stages with a growing bud exhibited much higher superoxide production than those with fully open flowers (stages four and five). However, in pollen from anthers of fully open flowers, the stained pollen numbers were very high (Fig.1), suggesting that developmental oxidative stresses are greatly enhanced in pollen after flower opening.

During pollen germination, on the other hand, there were no blue formazan deposits observable under a stereo microscope, implying no superoxide production. Unfortunately, in explaining this, there are no published reports, to date, suggesting whether or not NOXs might have a role in pollen tube tip growth. There has, however, been a study that seems to show that nitric oxide (NO), is involved in pollen tube growth and reorientation (Prado *et al.* 2004). According to this report, the rate and orientation of pollen tube growth is regulated by NO levels at the pollen tube tip. More specifically, it was found that endogenously produced NO is very low or absent at the tip of pollen tube and that there are higher levels behind the tip, where the postulated NO-producing peroxisomes are situated. This pattern of NO production allows pollen tube growth at the tip and plays a role as a positive feed back of extension in a straight growth axis. The study also found that the addition of NO donor SNAP (s-nitroso-acetylpenicillamine), which inhibits germination,

resulted in a dishevelled appearance in the medium, while pointed application of an NO donor near the pollen tube tip resulted in re-directed growth. It was postulated, therefore, that pollen germination is regulated not by superoxide production, but by NO levels at the pollen tube tip.

When changes in the superoxide production of pollen over a storage period (Plate 9A) were recorded, the pollen hardly showed any blue colour at all after 10 days of storage at room temperature, compared with the intense blue stained colour of fresh pollen, suggesting that there might be either a significant amount of superoxide dismutation or decreased superoxide production during the storage period. When dehiscent anthers containing pollen were stored, however, a decrease in SOD activity was observed (Fig. 2B). Therefore, it would appear that there was a decline in superoxide production, which might result from a decrease in the activity of NADPH oxidases during storage.

There are two possible explanations for the effect of heat (microwave treatments) on superoxide production of pollen (Plate 9B) showing that the heated pollen exhibited a yellow colour (no NBT reaction), in comparison to the intense blue colour exhibited by fresh pollen. One is the heat-related destruction of the NADP oxidases and peroxidase in the cell membrane or wall of pollen, which are presumably responsible for superoxide production. The other is an increase in SOD expression, as this can be activated by heat shock. The latter explanation has been illustrated by many reports that have shown that expression of the cytosolic Cu/Zn SOD is activated by paraquat, heat shock, chilling, ozone and salt stress (Tsang *et al.* 1991, Van Camp *et al.* 1996, Herouart *et al.* 1993). In one of these, it was found that cytosolic Cu/Zn SOD activity in *N. plumbaginifolia* is encoded by a single gene (SodCc). Utilizing a promoter- β -glucuronidase fusion of the SodCc gene, it was shown that the regulatory elements for the induction of cytosolic Cu/Zn SOD expression by paraquat, heat shock and chilling are included within a 744-bp fragment of the SodCc 5' flanking region (Van Camp *et al.* 1997). Moreover, it has also been demonstrated that microwave-induced stimulation can significantly boost SOD, L-DOPA (levo-dihydroxy phenylalanine) and phenolics in fava beans (*Vicia fava*), meaning that the phytopharmaceutical value was greatly improved (Randir and Shetty 2004). More specifically, this study found that, compared with a control, total SOD activity in all of the

microwave treated fava bean sprouts was much higher during germination, with the highest SOD activity found on days 1 and 7 of germination, showing a positive relationship with the antioxidant activity during that period. These results suggested that the high SOD activity on day 1 was consistent with increased L-DOPA synthesis and that the high SOD activity on day 7 corresponded to enhanced phenolics synthesis. It was therefore confirmed that microwave heat stress stimulated ROS, inducing and enhancing detoxifying antioxidant enzymes (such as SOD) as a response.

Although, in crude extracts from stage 5 anthers, SOD units/flower were not higher than at any other stage (Fig.4), superoxide was almost non-existent (Plate 4 and 5). It remains unclear whether the SOD activity was sufficient to keep the level of superoxide low, or whether superoxide production was reduced in stage 5 anthers.

This pattern, of higher SOD activity during the growth stages of petunia flower buds (Figs. 4, 5, 6 and 8), is reminiscent of the results reported in Norweign spruce trees (Polle *et al.* 1989), where young needles of Norwegian spruce trees exhibited higher SOD activity than mature and senescencing needles. In this research, the chlorophyll content at first increased with ageing, but then, after the initial phase of this process, remained constant. On the other hand the activity of SOD declined by roughly 25% within the needles over 3 to 4 years of ageing. This follows the findings of many other studies, which have also shown that the activity of SOD was found to be dependent on the developmental stage of the tissue. For example, in tomatoes, two Cu/Zn SOD genes were expressed at various developmental stages, and the flower buds and young leaves exhibited the highest levels of the two mRNAs (Perltreves and Galun 1991). Likewise, in cucumbers and pepper fruits (Rabinowitch and Sklan 1981), SOD activity levels were high in the immature green fruits of both species. Similar results were also obtained in the seedlings of *Acacia mangium*. In this study, phyllodes at different positions showed different total SOD and APX (ascorbate peroxidase), and total SOD and APX activities were highest at young phyllodes (at the apex), while they declined as the phyllodes matured (Yu and Ong 2000). It was postulated that the high activities of SOD and APX in the young phyllodes protected them during immaturity against high activities of ROS, resulting both from their high metabolic activities and from an excess of light. Conversely, the decreased activities of SOD and

APX in mature phyllodes appeared to be associated with their higher photosynthetic capacities, allowing these phyllodes to reduce oxidative stress.

Consistent with a number of studies which have demonstrated that high production of ROS induces high activities in detoxifying enzymes (Yu and Ong 2000, Randir and Shetty 2004) and that an increment of superoxide radical production induces SOD activity in plant cells (Bowler 1992), the research conducted in the present study also indicated a positive correlation between superoxide production and SOD activity at the five developmental stages. In anthers and filaments, the stages with the highest superoxide production (stages one and two) also showed high SOD activity, while those with low superoxide production (stages four and five) presented low SOD activity (Plates 5 and 6, Figs. 4 and 5). In the case of pollen (Figs. 1 and 7), however, superoxide production was comparatively high in the fully open flowers of stages four (predehiscent anthers) and five (dehiscent anthers), but while the SOD activity of stage four was high, that of stage five was lower. This is perhaps because, at anther dehiscence, pollen has high superoxide production when it is preparing to germinate and when it is released into the environment, where it is exposed to a significant number of detrimental agents such as UV light, pollutants, high and low temperatures and pathogenic attack. There have been comparatively very few studies on anti-oxidative mechanisms during the gametophytic phase of angiosperms, but Frova *et al.* (1989) did report that pollen can create new proteins under adverse environmental conditions, such as heat shock, especially when this occurs during the gametophytic phase. Therefore, an explanation of the lower SOD activity, compared with the high superoxide production, of mature pollen could be that it is related to the expression and activation of other antioxidants. This hypothesis is supported by a study that found a catalase gene expressed, activated and translated in the postmeiotic stage of mature maize pollen, while superoxide dismutase transcripts were present at low levels in the mature pollen. The CAT-1 isozyme in mature maize pollen was of gametophytic origin, and protein translation was consistent with the period of time over which pollen is normally released into a number of adverse environments. In this study it was thought that *Cat 1* expression could be of great importance for maize reproduction, because successful germination of the pollen grain under oxidative stresses is needed for transmission of the sperm cell's genetic complement and fertilization (Acevedo and Scandalios 1990).

In terms of the reaction of the male reproductive parts with the NBT solution at the five developmental stages of the petunia, the regions of blue formazan deposits have been found to show cells in which the amount of superoxide is significantly greater than the dismutation of superoxide (Fryer *et al.* 2002). Likewise, in the present study, a cross section of an anther at stage one (Plate 5) and a longitudinal section of a filament at stage five (Plate 7) both commonly exhibited a high amount of blue formazan deposits in their vascular bundles, suggesting that natural oxidative stress in the vascular bundle is very high. However, the part of the filament connected to the petal had no blue formazan deposits. This seems to be due to the vascular bundle probably being crushed when the stamen was pulled free from the petal. A vascular bundle, as a strand of conducting tissue, includes xylem, phloem and cambium. Moreover, the metabolic activity of vascular bundles in a natural developmental process is higher than that of any other tissue, because xylem and phloem are responsible for transporting water, dissolved nutrients and organic substances. It is therefore possible that oxidative stresses from this higher metabolic activity would result in higher superoxide production. By comparison, the work of Fryer *et al.* (2002) on photo-oxidative stress responses in leaves showed the majority of NBT staining in the mesophyll tissue and in particular numerous small NBT stained spots were found at the distal points of the microvasculature. This is because in this case, unlike in the present study, the staining is a response to photo-oxidative, rather than higher metabolic stress.

Furthermore, it was observed that, because cambium is meristematic, the metabolic activity of cambium in growing buds was also much higher than it was in mature flowers. This was particularly apparent in the cambium, known as fascicular cambium, located inside the vascular bundles. Moreover, the vascular bundles themselves are joined by sections of parenchyma cells and, as these parenchyma cells are also meristematic, interfascicular cambium is also formed. It has already been demonstrated that this fascicular and interfascicular cambium forms a continuous cylindrical layer, and that its meristematic activities result in secondary thickening (Bowes 1996). In our study, a comparison of cross sections of anthers between stage one and five (Plate 5) showed that the vascular bundles of growing buds exhibited intense blue formazan deposits, suggesting that the higher levels

of superoxide production were a result of the higher metabolic activity needed for growth.

This high superoxide production in the vascular bundle of a petunia anther should also be expected to exhibit equally high SOD activity (Bowler *et al.* 1992), consistent with the indication of high SOD activity, particularly that encoded by the Cu/Zn SOD gene in the vascular bundle of transgenic tobacco anthers (Herouart *et al.* 1994). In order to study the developmental and environmental regulation of Cu/Zn SOD, Herourat *et al.* (1994) used the promoter of the *Nicotiana Plumbaginifolia* gene encoding cytosolic Cu/Zn SOD (SODCc), and this was fused to the β -glucurodinase (GUS) reporter gene (*gusA*). In this transgenic tobacco flower, GUS activity was found in the vascular tissue of ovaries and anthers, in ovules, and in pollen grains. It is noticeable that the promoter of this transgenic tobacco flower was activated in the vascular bundles of anthers, correlating with a high level of superoxide production in the vascular bundles of anthers found in the present study (Plate 5). It is also significant that almost half of the pollen grains exhibited GUS activity in the transgenic tobacco, while, similarly, nearly half of the pollen grains in the petunia flowers in the present study were stained with NBT solution (Fig.1). The expression pattern of the SODCc-*gusA* gene in the transgenic tobacco flower was, however, somewhat different from the SOD activity in the present study (Figs. 4 – 8), in that GUS activity was higher in mature flower tissue than in flower bud tissue. In transgenic tobacco flowers, on the other hand, only dehiscent anthers, at various developmental stages, showed GUS activity. These different expressions of SOD activity at various developmental stages are explicable in two ways. One possibility is that the transcriptional regulation at the cellular level could be different from the natural expression at the protein level. The other is that the developmental regulation of Cu/Zn SOD by itself in transgenic tobacco flowers might be different from developmental SOD activities that include Cu/Zn SOD, Mn SOD and Fe SOD in petunia flowers.

Superoxide production in petunia stamens at various developmental stages in our research also exhibited a similar pattern to the tissue-specific pattern of *SodA2* promoter activity in the anthers of transgenic tobacco in another earlier report (Van camp *et al.* 1996). In the latter study, two Mn SOD genes from *Nicotiana plumbaginifolia* were isolated, and the 5' upstream regulatory region of these genes was fused to the β -glucuronidase reporter gene.

The result of these two gene fusions was the presentation of tissue-specific activity in transgenic tobacco and, particularly, the expression of Mn SOD chimeric gene changes in anthers during flowering. When the flower buds were young, for example, it appeared that promoter activity for the *SodA2* gene fusion was found in the vascular bundle. In the later stages, however, the GUS activity declined in the vascular tissue, but GUS activity in pollen was developed and maintained, at least until dehiscence. This pattern is exactly consistent with the occurrence in the present study of superoxide production in petunia anthers (Plate 5) and pollen (Fig 1). Furthermore, this conclusion is also consistent with the research of Bowler *et al.* (1994), in which the major sites of superoxide production correspond to the location of SODs in the cell. On the other hand, the promoter activity of *SodA1*, when the flower buds were young, was somewhat different from that of *SodA2*, in that the GUS activity of the anthers was not present in the vascular bundle. There were, however, also some similarities between the activity of the *SodA1* gene fusion, during further anther development, and the activity of *SodA2*, in that GUS activity showed in mature pollen. In other words, the promoter activity of both the *SodA1* and the *SodA2* was expressed simultaneously in mature pollen, implying that there could be a high rate of mitochondrial superoxide production because of dramatically increased respiration during anthesis stress (Zhu and Scandalios 1993).

In general, therefore, the regulation of SOD activity throughout the developmental stages of petunia stamens exhibits strong similarities with the pattern of SOD studied at the gene expression level in the tissues of tobacco. It must be remembered, however, that there have been relatively few reports to date showing SOD activity within flower tissues. Therefore, the difference in the expression and regulation of SOD activity between transgenic tobacco flowers and natural petunia flowers may also result from differences in analysis level, the kinds of isozymes included in the study, and any tissue-specific activity from those isozymes.

4.3 Analysis of SOD isozymes in the crude extracts of male reproductive parts of petunia flower

A native PAGE analysis was performed using a vertical slab gel apparatus and SOD isozymes were localized by the photochemical procedure reported by Beauchamps and Fridovich (1971). The insolubility of the blue reduction product of NBT was used to detect superoxide dismutase on polyacrylamide gels. This method directly detects SOD isozymes after polyacrylamide electrophoresis, and also separates isozyme bands in order of increasing relative mobility. In this case, electrophoresis indicated that the crude extracts from anthers, filaments, stamens and pollen commonly had three kinds of isozymes at all developmental stages. The differences that these isozymes had in their sensitivity to certain inhibitors (namely, cyanide and H_2O_2), showed that they were Cu/Zn SOD, Fe SOD and Mn SOD. These results are the first reported case of three different SOD isozymes in a flower tissue. However, this may be because, until now, there has been very little known about SODs in flower tissues in general. In the literature, the most comparable results to date have been Cu/Zn SODs being reported in olive pollen (Alche *et al.* 1998) and camellia pollen (Xiao-hong *et al.* 2005), Mn SOD being reported in tobacco floral nectar (Carter and Thornburg 2000), and Cu/Zn SODs and Mn SODs being reported in the pollen and anthers of *Zea mays* (Oden *et al.* 1992). Similarly, Fe SOD has rarely been reported in higher plants. While some researchers have found that certain plant species contain Fe SOD in their chloroplasts and peroxisome (Arora *et al.* 2002), a number of plants including tomatoes (Perltreves and Galun 1991), watermelon cotyledons (Bueno and Delrio 1992), and *Radix lethospermi* seeds (Haddad *et al.* 2005) have been found to contain only Cu/Zn SODs. It has, however, also been reported that a few higher plants, for example, maize (Baum 1981) and wheat (Wu *et al.* 1999), have both Mn SODs and Cu/Zn SODs.

The activities of the three different SODs found in petunia stamens are not under independent developmental controls. This can be concluded from the evidence showing that crude extracts from anthers, filaments, stamens and pollen all commonly exhibit the three different kinds of isozymes detectable after native PAGE throughout all developmental stages. This is, therefore, somewhat different to the findings of Kroniger *et*

al. (1993), which showed that two Cu/Zn SODs and Mn SODs, in the seedlings and needles of Norway spruce, appeared or disappeared independently during a particular developmental stage. Kroniger's work suggested that while SOD II (Cu/Zn SOD) was dominant in seeds and foliar buds, SOD I (Cu/Zn SOD) was abundant in mature needles, and that SOD III (Mn SOD) disappeared during the bud break and reappeared in mature needles.

It was observed that the same active bands on native isozyme gels from different tissues were found to have obvious quantitative differences, depending on the particular stage of development. The findings from the SOD activity assays are consistent with this. All male reproductive parts of petunia at those developmental stages with high SOD unit/flower also had isozymes bands with strong activity staining. In addition, with the exception of crude extracts from pollen with high SOD activity and strong activity staining at stage four, there were major similarities of SOD patterns in all tissues, as isozyme bands with relatively strong activity staining were present at stages one to three, while those with lower activity staining were present at stage five (compare the results in Figs. 4 to 7 with those in Plate 11).

Furthermore, when examining the gels, it was also found that the pattern of SOD isozymes from all of the tissues at the five developmental stages (Plate 11) had much in common. The first (Mn SOD) and second (Fe SOD) bands were more intense than the third (Cu/Zn SOD), with the third (Cu/Zn SOD) bands in pollen being especially weak, suggesting that the activities of Mn SOD and Fe SOD in the crude extracts were higher than Cu/Zn SOD. These results are very different compared to those in the published research. Oden *et al.* (1992), for example, reported that Cu/Zn SOD is the most abundant SOD in higher plants, and some other studies have reported that Mn SOD showed only 3 - 5% of total SOD activity in some plants (Fridovich 1986) and 1 – 4% of total SOD activity in needles of Scots pine trees (Wingsle *et al.* 1991). Moreover, patterns of SOD isoenzymes in pea leaves (del Rio *et al.* 2003) and Scots pine seeds (Streller 1994) on native PAGE gel showed that Mn SOD bands were much weaker than those of Cu/Zn SODs, and in some other studies, the activity of Mn SOD sometimes could not even be confirmed by a visible band (Wingsle *et al.* 1991). Likewise, Fe SOD is not expressed to detectable levels in some

other plant species such as Norway spruce (Kroniger *et al.* 1993) and bean (Pitcher *et al.* 1992)

4.4 Purification of superoxide dismutase

SOD from 1,000 stamens at stage five was partially purified using ammonium sulphate fractionation and ion exchange chromatography. The purified fraction accounted for 3% of total activity and 0.83% of total protein, compared with crude extracts.

This process of purification was conducted by adding ammonium sulphate in three steps (0 - 30%, 30 - 60% and 60 - 90%) to remove unwanted proteins in the crude extracts. After the third addition (60 - 90%) and then retaining the supernatant, 4.5 fold purity with 13.5% yield of SOD activity was achieved. In the following ion exchange chromatography step, no high peak at absorbance (280 nm) was found. This could have been due to a very low protein content (0.627 mg) loaded to the DEAE cellulose column. The first modification attempted was the use of a longer time-frame (over 24 hours at 4 °C) to precipitate the proteins with the same percentage of ammonium sulphate, but this was not successful. Therefore, one step additions were then attempted, and 0 - 40% saturation with ammonium sulphate was selected before the ion exchange chromatography step (Fig. 9). This gave two distinguishable high peaks in the elution profile of the DEAE cellulose column - one in the unbound fractions, the other in the bound protein fractions. The bound fraction with the highest peak was confirmed on a native PAGE gel to be purified with only one isozyme (Plate 13).

Partial fractionation with ammonium sulphate (0 - 40 %) and dialysis of the crude extracts did not change the banding patterns (Plate 13). Unbound fractions, after partial purification by ion exchange chromatography, still had three isozyme bands on the native gel, but the first band became fainter (less SOD activity staining), suggesting that the first band of isozymes had been adsorbed on the DEAE cellulose in the ion-exchange column and were purified as bound fractions. Furthermore, there seems to be an absence of substantial enzyme modification during the enzyme purification procedure (Plate 15 C). The bound

fractions, with only one isozyme (first band), could be identified as a Mn SOD, because this is resistant to both potassium cyanide and H_2O_2 . This result is reminiscent of the response in the transgenic tobacco report by Van Camp *et al.* (1996). This study found that, in transgenic tobacco flowers, two Mn SOD gene fusions displayed tissue specificity, as both promoter::GUS fusions were expressed simultaneously in mature pollen. Taking into account that this study used 1,000 stamens from the fifth stage (fully open dehiscent flower) in the process of purification, it is possible that a considerable proportion of the purified Mn SOD might come from mature pollen.

The specific activity (66.5 U/mg) of the purified isozyme, which was confirmed as Mn SOD by the reaction of inhibitors, was three times higher than that of crude extracts (Table 3). However, even though the same purification steps were followed, the specific activity of the purified SOD was much lower than that reported for purified Cu/Zn SODs from *Radix lethospermi* seeds (Haddad and Yuan 2005) and tobacco leaves (Ragusa *et al.* 2001). On the other hand, the specific activity of the purified Mn SOD, compared with Mn SOD from Norway spruce (Kroniger *et al.* 1995) which is 322 U/mg, was a little lower. While low specific activity may just be a characteristic of Mn SODs from higher plants (Baum and Scandalios 1981), there are several possible explanations for the very low specific activity of purified Mn SOD. Firstly, some parts of SODs undergo a process of inertia during the longer purification procedures, because enzymes are sensitive to experimental environments and delayed time. In this study, the purification procedure - including ammonium sulphate fractionation, dialysis, concentration and elution processes for ion exchange chromatography - took over 48 hours. Secondly, the loss of Mn from Mn SOD during the longer purification procedure could decrease the specific activity of Mn SOD (Streller *et al.* 1994). This effect was also reported by Hayakawa *et al.* (1985), who described a loss of Mn from Mn SOD during the purification of the enzyme from spinach. Thirdly, there could also have been a possibility that the azide added in elution buffer as an anti-bacterial reagent might have had an inhibitor effect on Mn SOD activity. According to one report (Sevilla 1982 *et al.*), 35 mM and 10 mM azide inhibit Mn SOD activity in the pea enzyme extracts more than 60% and 38%, respectively. The concentration of azide in the present study is very low (3 mM), but a minor possibility of an inhibition by the azide used could not be ruled out.

In this study, Mn SOD was purified 3.7 fold with a 3.0% yield from the petunia stamen. Before this study, there have been few reported purifications of Mn SOD from higher plant sources. In one of these, Mn SOD was purified 444 fold, with a 1% yield, from the Norway spruce seeds in a three step purification process - comprising ammonium sulphate fractionation (30 - 90%), anion-exchange chromatography and gel filtration on Superose 12 (Kroniger *et al.* 1995). Another example from a higher plant, *Pisum sativum*, involved Mn SOD being purified 215 fold, with 1.7% yield, in steps that comprised thermal fractionation, ammonium sulphate salting out (40 - 70%), ion exchange and gel filtration chromatography, and preparative polyacrylamide gel electrophoresis (Sevilla *et al.* 1980). Mn SOD has also been purified 172 fold, with 10% yield, from germinating seeds of Scots pine. In this case, the purification procedure involved 30% ammonium sulphate fractionation, anion-exchange and hydrophobic-interaction chromatographies, and chromatofocusing (Streller *et al.* 1994).

Compared with all of the Mn SODs purified from other plant sources, the Mn SOD from petunia stamens was purified from other isozymes after only three steps. All Mn SODs purified from plant sources, including the petunia stamens in this study, commonly had low yields. One explanation for these poor recoveries could be the specifically higher instability of Mn SOD in comparison to, for example, Cu/Zn SOD. This instability has been ameliorated in peas through the addition of cysteine in some of the steps of the purification process to enhance Mn SOD stability (Sevilla *et al.* 1980). A further explanation, however, for the low yields may be due to the occurrence of some interfering substances in the crude extracts, for example, Mn-pigment-protein complexes (Von Kamake and Wegmann 1977) and metal ions (Lumsden and Hall 1975).

4.5 Characterization of purified enzymes

After ion exchange chromatography, two major bands of 57,000 and 26,000 Da, according to the distances migrated on SDS-PAGE gel, were distinguished. All purified Mn SODs previously reported from higher plants, with the exception of thylakoid-bound Mn SOD from spinach, which had a subunit molecular weight of 26,000 Da (Hayakawa *et al.* 1985),

are tetramers with subunit molecular weights of 22,000 – 27,000 Da and molecular weights of 86,000 – 98,000 Da (Sevilla *et al.* 1980, Streller *et al.* 1994, Kroniger *et al.* 1995). Therefore, the size of the second band in our study is in agreement with the molecular masses reported for Mn SOD subunits from other plant sources. There is, however, a need for further research that focuses on progressive purification procedures, such as those using the Sephadex column, to purify the enzyme to apparent homogeneity, as this would allow for a more comprehensive study of the accurate molecular weights and other characteristics of this purified enzyme.

The activity of the purified enzyme was investigated over a pH range from 3.0 to 10.0 (Fig. 11A). It is clear that SOD activity in the bound fraction was optimal in the pH range of 7 – 7.8 and was at its highest at 7.8. In other words, the purified enzyme was very active in neutral solutions, which is in accordance with the impact of pH in crude extracts (Fig. 3A), suggesting that the properties of SOD did not greatly change after purification and the Mn SOD activity could be very high in the crude extracts. The effect of higher levels of pH on the purified enzyme in this research (Fig. 11 A) is also in accordance with other studies, which show that Mn SOD activities in purified enzymes decrease at pH values higher than 7.8 (Almansa *et al.* 1994). In general, all SODs studied to date have been reported to be very active at neutral pH, but the range of optimum pH values for Mn SOD activities has usually been demonstrated to be narrower than that of Cu/Zn SODs (Kroniger *et al.* 1995). For example, the pH value of purified Cu/Zn SOD from *Radix lethospermi* seed retained most of its activity in the range of 6.0 – 10.0 (Haddad and Yuan 2005), while purified Fe and Mn SODs presented gradual decrease at alkaline pH values (Sevilla *et al.* 1982, Almansa *et al.* 1994). Particularly similar to the results of our study were those reported for the activities of Mn SODs from maize, as these decreased considerably at pH 10 (Baum and Scandalios 1981). The effect of pH on the stability of the purified enzyme was also tested through incubation at various pH levels for 24 hours, and the purified fraction retained more than 90% activity around a pH range of 7 to 8.5 and favourable stability was confirmed (Fig. 11B).

Results from the study of thermal stability during a storage period indicated that the purified enzyme, like that in the crude extracts, was also stable at –20 °C for at least four

weeks (Fig.12). After four weeks at -20°C , 87% of its initial activity remained, but at 4°C only 38% of the initial activity remained. These results were similar to the SOD activities of crude extracts and dehiscent anthers at different storage periods and temperatures (Fig. 2), suggesting that SOD properties were not greatly modified after purification. Similar studies have also been reported. For example, purified Mn SOD from a higher plant, *Pisum sativum*, retained 82% and 76% of its initial activity at 4°C and -20°C , respectively, after storage of one month (Sevilla *et al.* 1980). In another study, purified Fe SOD from a higher plant, *Citrus Limonum*, was very stable at low temperatures. The purified enzyme had 81% and 86% of its initial activity after eight months at -20°C and one month at 4°C , respectively (Almansa *et al.* 1994).

Unfortunately, more detailed and comprehensive investigation into the characteristics of the purified enzyme could not be performed because of the present limitations in time and resources. To enable a better understanding of these characteristics, future studies should utilize a wider range of procedures - such as metal analysis, spectroscopic characterization and amino acid analysis.

4.6 Physiological role and biomedical significance

It is thought that SODs could play an important role in reproductive physiology as protectors against oxidative stress. A number of the results from this study suggest that the anti-oxidative functions of SODs in the male reproductive physiology of petunia flowers are important. Firstly, there are the findings that while SODs are produced at all developmental stages of the stamen, there are also higher levels of SOD activity at the developmental stages with higher superoxide production (the stages with higher metabolic activity). Secondly, it is also significant that most SOD activity is concentrated in the anthers, which produce pollen, rather than in the filaments, which only have a support function. Thirdly, the presence of three kinds of SODs in pollen grains strongly suggests a physiological role as a protective mechanism against oxidative stress during the stages of pollen development, depending on the haploid programme of gene expression. Finally, it appears important that petunia stamen have three different forms of SODs, in contrast to the types of SODs found in other higher plants, because this may provide the petunia with

more effective defence mechanisms than those of many other plants. In angiosperms and gymnosperms, Cu/Zn SODs are the most important isoenzymes, and a number of plants have only one type of SOD (Kanematsu 1989, Bridges and Salin 1981). In these plants, hydrogen peroxide, which is produced by the dismutation reaction, may gradually inhibit the cytosolic enzymes (Baum and Scandalios 1981). This is also consistent with Tepperman and Dunsumuir (1990) who, when producing transgenic tobacco plants, suggested that elevating Cu/Zn SOD alone could not protect against oxygen toxicity, which means that enhancing the other enzymes involved in H_2O_2 detoxification should be of great importance. Also, the existence of a hydrogen peroxide-resistant Mn SOD enzyme may offer more effective protection against oxidative stress.

The effect that the SODs present in the petunia stamen appear to have on superoxide radical-anions may be useful for pharmaceutical applications. In order to be accepted for wide-spread use in the pharmaceutical industry, however, new technologies for the production and purification of the enzymes will be required. The recent development of immobilized metal affinity chromatography (IMAC) has provided a new and well-defined technology for enzyme purification (Michalski 1996, Wu *et al.* 2006), and might also be a solution to this problem.

Moreover, as a result of recombinant DNA technology, the SODs found in pollen can now also be used for clinical purposes. For example, Ole e 5 is the first Cu/Zn SOD distinguished as an allergen from *Olea europaea* pollen. The cloning and expression of Ole e 5 in a perfect form in *E. coli* could be an effective tool for studying allergen cross-reactivity between pollen and allergenic sources (Butteroni *et al.* 2005). When considering that pollen is a major cause of allergy and allergenic diseases, this type of pollen study has to be extended to petunia as well as other plants. Because more production of recombinant allergen from various kinds of plant sources and their use can help to enhance comprehension of allergen, to define families of allergens, and to investigate allergenic cross-reactions. To date, 27 recombinant allergens from 7 different allergenic sources have been developed and used for clinical and scientific purposes (Metz-Favre *et al.* 2006). In particular, as petunia is a plant grown worldwide, cloning and expression of recombinant allergens from petunia could help a number of allergenic patients in diagnosis and

therapeutic decision.

Another possible application of this study's findings comes about through a comparison with the occurrence of SODs in the pollen and anthers of *Zea mays*. The Cu/Zn- and Mn SODs which occur in extracts from the pollen and anthers of *Z. mays* are used in two commercial products - Baxtin and Polbax (Oden *et al.* 1992). It would be interesting to see if petunia pollen and anthers could substitute for these *Z. mays* pollen and anthers extracts.

The three kinds of SODs found in the petunia's anthers and pollen have also been established to be of value in the treatment of many pathological states in humans. For example, Orgotein is a pharmaceutical version of refined Cu/Zn SOD, used for its anti-inflammatory and antiviral activities (Escribano *et al.* 2002). In fact, pollen and pollen extracts from several plant sources are already in use for their anti-inflammatory effects in humans. It seems reasonable to assume, therefore, that the application of petunia pollen, with its three different forms of SODs compared to other pollen with only Cu/Zn SODs, to pathological states would be even more effective.

4.7 Direction for future studies

This study examined superoxide production and SOD activities at five developmental stages of the petunia stamen, and then the Mn SOD was partially purified after ammonium sulphate fractionation and ion exchange chromatography. In the course of this research, it was found that petunia anthers and pollen include three types of SODs and have high SOD activities. Future study in this area, therefore, needs to focus on further purification of the SODs, and a more detailed characterization of them, by using a wider range of purification procedures. This can be accomplished by the use of additional steps - such as cation-exchange, chromatofocusing and size exclusion chromatography. Moreover, the IMAC method - which has been used for the purification of commercial Fe, Mn and Cu/Zn SODs to create specific antibodies - is capable of the resolution of Fe, Mn and Cu/Zn SODs in one column. In this method, SODs can be purified and eluted successively and effectively from a Cu²⁺ IMAC column, with a raising gradient of a counter ion run in combination with a raising pH gradient (Michalski 1996, Wu *et al.* 2006). In the present study, it is

noticeable that there is an occurrence of Fe SOD, confirmed by strong bands on native PAGE. In the future studies, it could be valuable to focus on the purification and more extensive characterization of the Fe SOD.

When considering that microwave-induced stimulation in fava bean (Randir and Shetty 2004) greatly enhanced SOD and pharmaceutical value, it is possible that enhanced superoxide dismutation in anthers and pollen could have resulted from microwave treatment (Plate 9B). In future studies, more detailed SOD assay, characterization, and changes of other antioxidant activities need to be investigated.

Another important area of future research is in immunology. Antigenic specificities for the three SOD isozymes can be identified by preparing antibodies against each of the three isozymes, which could also help to further characterize the petunia SODs.

The promoter of the *Nicotiana plumbaginifolia* gene encoding SOD has been fused to the β -glucuronidase (GUS) reporter gene, and the subsequent analysis of the promoter activity has offered information about transcriptional regulation at the cellular level in transgenic tobacco plants (Herouart *et al.* 1994). Recently, as applications of this study, a number of transgenic plants have been created and helped to study anti-oxidative mechanisms. For example, transgenic cucumber fruits with elevated levels of an anti-aging SOD (Lee *et al.* 2003), transgenic *Arabidopsis* plants enhanced with salt tolerance (Wang *et al.* 2004), and transgenic rice plants with highly enhanced drought tolerance (Wang *et al.* 2005) have been created. Likewise, for better insights about the developmental and environmental regulation of petunia SODs, genes encoding the three SOD isoforms present in the petunia need to be isolated and characterized. Furthermore, cloning SOD genes from the petunia, and creating transgenic plants from these, would help in the study of the regulation and function of SODs in response to various oxidative stresses in the reproductive tissues of higher plants.

4.8 Conclusions

The result of this study shows that SOD activities are concentrated in anthers and pollen. It was also found that SODs play a significant role in protecting the reproductive tissue against oxidative stress by maintaining a sophisticated balance between levels of $O_2^{\cdot-}$ generation and removal. It can, therefore, be postulated that high superoxide production in male reproductive tissue during pollen development requires a high level of SOD activity in order to reduce oxidative damage and prevent genetic harm to gametophytic tissues.

It is noticeable that there are occurrences of three different SOD isozymes at all developmental stages in crude extracts of male reproductive tissues on native PAGE gel, and Mn SOD and Fe SOD isozyme bands presented stronger activity staining, suggesting higher SOD activities, than Cu/Zn SOD bands. Compared with most higher plants with only Cu/Zn SODs, the abundant existence of hydrogen peroxide-resistant Mn SOD in petunia implicates more effective anti-oxidative mechanisms. Moreover, after partial purification and characterization of a Mn SOD from the fifth stage stamen with dehiscent anther, its properties were similar to those of the crude extracts, suggesting Mn SOD activity is very high in the crude extracts. When considering tissue-specific activity of two Mn SOD promoters in transgenic tobacco (Van Camp *et al.* 1996), especially their simultaneous expression of Mn SOD activities in mature pollen, it is postulated that mitochondrial superoxide production is most elevated during anthesis which, in turn, results in a high level of Mn SOD activity.

There has been comparatively little research on mechanisms against oxidative stress in reproductive tissues of higher plants. The existence of three different kinds of isozymes, and the preliminary characterization of the SODs from petunia anthers and pollen, could offer a useful starting point towards further study of SOD activities in reproductive mechanisms and potentially affect many areas, ranging from plant biotechnology to biomedicine.

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APPENDICES

APPENDIX-A

1.1 Buffers

1.1.1 0.1 M Potassium phosphate buffer (pH 7.8) : Extraction Buffer

Solution A : K_2HPO_4 17.418 g/100 ml

Solution B : KH_2PO_4 13.609 g/100 ml

Solution A (9.2 ml) was mixed with solution B (90.8 ml) and brought to 1 L with dH_2O .

1.1.2 0.1 M Citrate phosphate buffer (pH 3.0 to pH 7.0)

Solution A : 0.1 M solution of citric acid 19.21 g in 1000 ml

Solution B : 0.2 M solution of dibasic sodium phosphate

53.65 g of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ in 1000 ml

x ml of A + y ml of B, diluted to a total of 100 ml

x	y	pH
39.8	10.2	3.0
30.7	19.3	4.0
24.3	25.7	5.0
17.9	32.1	6.0
6.5	43.6	7.0

1.1.3 pH 8.5 to pH 10.0 buffer

25 mM acetic acid (Sodium Acetate) 12.5 ml

25 mM MES 12.5 ml

50 mM Trizma base 25.0 ml

The mixture was brought to 100 ml with dH_2O and the pH was adjusted by 1M HCL or 1M KOH.

1.2 Bradford Reagent (Bradford 1976)

Coomassie Brilliant Blue G-250	100 mg
Ethanol (95% v/v)	50 ml
Phosphoric acid 85% (w/v)	100 ml

The Coomassie Brilliant Blue G-250 was dissolved in ethanol. This was mixed with phosphoric acid and the solution was brought to 1L with dH₂O before it was vacuum filtered and left to settle for three days before it was used and then kept in a dark bottle at a room temperature.

APPENDIX-B

1.1 Reagents and gel preparation for native PAGE gels

(from the Bio Rad Mini – Protean Dual Slab Cell Instruction Manual)

1.1.1 Separating gel (12.0%)

DH ₂ O	3.45 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml
30% acrylamide : bis solution	4.0 ml
10% ammonium persulphate (w/v)	50 µl
TEMED	5 µl

1.1.2 Stacking gel

DH ₂ O	6.2 ml
0.5 M Tris-HCl, pH 6.8	2.5 ml
30% acrylamide : bis solution	1.3 ml
10% ammonium persulphate (w/v)	50 µl
TEMED	10 µl

1.1.3 Running Buffer (pH 8.3)

Trizma base	3 g
Glycine	14.6 g

The mixture was dissolved in 150 ml of dH₂O and it was brought to 200 ml.

Before use, 60 ml stock + 240 ml dH₂O.

1.1.4 1.5 M Tris-HCl, pH 8.8

27.23 g of Trizma base (18.15 g/100 ml) was dissolved in 80 ml of dH₂O and it was adjusted to pH 8.8 with 1 N HCl. Then the solution was brought to 150 mL with dH₂O and it was stored at 4 °C.

1.1.5 0.5 M Tris-HCl, pH 6.8

6 g Tris base was dissolved in 60 ml of dH₂O and it was adjusted to pH 6.8 with 1 N HCl. Then the solution was brought to 100 ml with dH₂O and it was stored at 4 °C.

1.1.6 30% acrylamide :bis solution

87.6 g Acrylamide (BioRad) 29.2 g/100 ml

2.4 g N'N'-bis-methylene-acrylamide 0.8 g/100 ml

The mixture was brought to 300 ml with distilled water.

Then it was filtered and stored at 4 °C in the dark.

1.1.7 Staining for SOD isozymes

step1: Incubate gel in 20 ml NBT (4.1 mg in 50 ml dH₂O) for 20 minutes.

step2: Illuminate gel in 12 ml of mixture for 24 hours.

The mixture consists of 0.01 M EDTA 800 µl, 0.13 mM riboflavin 200 µl, 0.1 M potassium phosphate buffer 11 ml.

step3: Rinse with distilled water.

APPENDIX-C

1.1 Reagents and gel preparation for SDS PAGE gels

(from the Bio Rad Mini – Protean Dual Slab Cell Instruction Manual)

1.1.1 Seperating gel (12.0%)

DH ₂ O	3.35 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml
10% (w/v) SDS stock	100 µl
30% acrylamide : bis solution	4.0 ml
10% ammonium persulphate (w/v)	50 µl
TEMED	5 µl

1.1.2 Stacking gel

DH ₂ O	6.1 ml
0.5 M Tris-HCl, pH 6.8	2.5 ml
10% (w/v) SDS	100 µl
30% acrylamide : bis solution	1.3 ml
10% ammonium persulphate (w/v)	50 µl
TEMED	10 µl

1.1.3 Running buffer

Trizma base	9 g
Glycine	43.2 g
SDS	3 g

The mixture was brought to 600 ml with dH₂O and the solution was stored at room temperature.

Before use, 60 ml stock + 240 ml dH₂O.

1.1.4 1.5 M Tris-HCl, pH 8.8

27.23 g of Trizma base (18,15 g/100 ml) was dissolved in 80 ml of dH₂O and it was adjusted to pH 8.8 with 1 N HCl. Then the solution was allowed to 150 mL with dH₂O and it was stored at 4 °C.

1.1.5 0.5 M Tris-HCl, pH 6.8

6 g Tris base was dissolved in 60 ml of dH₂O and it was adjusted to pH 6.8 with 1 N HCl. Then the solution was brought to 100 ml with dH₂O and it was stored at 4 °C.

1.1.6 Sample buffer

dH ₂ O	4.0 ml
0.5 M Tris-HCl, pH 6.8	1.0 ml
Glycerol	0.8 ml
10% (w/v) SDS	1.6 ml
2-mercaptoethanol	0.4 ml
0.05% (w/v) bromophenol blue	0.2ml

The sample was diluted with sample buffer (4:1) and it was boiled for 5 minutes.

1.1.7 Stain and Destain**-Coomassie Blue Stain**

Methanol	40 ml
Glacial acetic acid	10 ml
Coomassie Brilliant Blue R250	0.1 g
dH ₂ O	50 ml

-Destain

Glacial acetic acid	10 ml
Methanol	40 ml
dH ₂ O	150 ml